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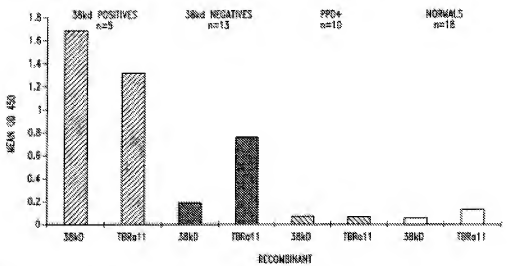

(57) Abstract

Compounds and methods for diagnosing tuberculosis are disclosed. The compounds provided include polypeptides that contain at least one antigenic portion of one or more *M. tuberculosis* proteins, and DNA sequences encoding such polypeptides. Diagnostic kits containing such polypeptides or DNA sequences and a suitable detection reagent may be used for the detection of *M. tuberculosis* infection in patients and biological samples. Antibodies directed against such polypeptides are also provided.

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COMPOUNDS AND METHODS FOR DIAGNOSIS OF TUBERCULOSIS

TECHNICAL FIELD

The present invention relates generally to the detection of *Mycobacterium tuberculosis* infection. The invention is more particularly related to polypeptides comprising a *Mycobacterium tuberculosis* antigen, or a portion or other variant thereof, and the use of such polypeptides for the serodiagnosis of *Mycobacterium tuberculosis* infection.

BACKGROUND OF THE INVENTION

Tuberculosis is a chronic, infectious disease, that is generally caused by infection with *Mycobacterium tuberculosis*. It is a major disease in developing countries, as well as an increasing problem in developed areas of the world, with about 8 million new cases and 3 million deaths each year. Although the infection may be asymptomatic for a considerable period of time, the disease is most commonly manifested as an acute inflammation of the lungs, resulting in fever and a nonproductive cough. If left untreated, serious complications and death typically result.

Although tuberculosis can generally be controlled using extended antibiotic therapy, such treatment is not sufficient to prevent the spread of the disease. Infected individuals may be asymptomatic, but contagious, for some time. In addition, although compliance with the treatment regimen is critical, patient behavior is difficult to monitor. Some patients do not complete the course of treatment, which can lead to ineffective treatment and the development of drug resistance.

Inhibiting the spread of tuberculosis will require effective vaccination and accurate, early diagnosis of the disease. Currently, vaccination with live bacteria is the most efficient method for inducing protective immunity. The most common Mycobacterium for this purpose is Bacillus Calmette-Guerin (BCG), an avirulent strain of *Mycobacterium bovis*. However, the safety and efficacy of BCG is a source of controversy and some countries, such as the United States, do not vaccinate the general public. Diagnosis is commonly achieved using a skin test, which involves intradermal exposure to tuberculin PPD (protein-purified derivative). Antigen-specific T cell responses result in measurable incubation at the injection

site by 48-72 hours after injection, which indicates exposure to Mycobacterial antigens. Sensitivity and specificity have, however, been a problem with this test, and individuals vaccinated with BCG cannot be distinguished from infected individuals.

While macrophages have been shown to act as the principal effectors of *M. tuberculosis* immunity, T cells are the predominant inducers of such immunity. The essential role of T cells in protection against *M. tuberculosis* infection is illustrated by the frequent occurrence of *M. tuberculosis* in AIDS patients, due to the depletion of CD4 T cells associated with human immunodeficiency virus (HIV) infection. Mycobacterium-reactive CD4 T cells have been shown to be potent producers of gamma-interferon (IFN-γ), which, in turn, has been shown to trigger the anti-mycobacterial effects of macrophages in mice. While the role of IFN-γ in humans is less clear, studies have shown that 1,25-dihydroxy-vitamin D3, either alone or in combination with IFN-γ or tumor necrosis factor-alpha, activates human macrophages to inhibit *M. tuberculosis* infection. Furthermore, it is known that IFN-γ stimulates human macrophages to make 1,25-dihydroxy-vitamin D3. Similarly, IL-12 has been shown to play a role in stimulating resistance to *M. tuberculosis* infection. For a review of the immunology of *M. tuberculosis* infection *see* Chan and Kaufmann, in *Tuberculosis: Pathogenesis, Protection and Control*, Bloom (ed.), ASM Press, Washington, DC, 1994.

Accordingly, there is a need in the art for improved diagnostic methods for detecting tuberculosis. The present invention fulfills this need and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides compositions and methods for diagnosing tuberculosis. In one aspect, polypeptides are provided comprising an antigenic portion of a soluble *M. tuberculosis* antigen, or a variant of such an antigen that differs only in conservative substitutions and/or modifications. In one embodiment of this aspect, the soluble antigen has one of the following N-terminal sequences:

(a) Asp-Pro-Val-Asp-Ala-Val-Ile-Asn-Thr-Thr-Cys-Asn-Tyr-Gly-Gln-Val-Val-Ala-Ala-Leu (SEQ ID NO: 115);

(b) Ala-Val-Glu-Ser-Gly-Met-Leu-Ala-Leu-Gly-Thr-Pro-Ala-Pro-Ser (SEQ ID NO: 116);

(c) Ala-Ala-Met-Lys-Pro-Arg-Thr-Gly-Asp-Gly-Pro-Leu-Glu-Ala-Ala-Lys-Glu-Gly-Arg (SEQ ID NO: 117);

(d) Tyr-Tyr-Trp-Cys-Pro-Gly-Gln-Pro-Phe-Asp-Pro-Ala-Trp-Gly-Pro (SEQ ID NO: 118);

(e) Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Gln-Xaa-Ala-Val (SEQ ID NO: 119);

(f) Ala-Glu-Glu-Ser-Ile-Ser-Thr-Xaa-Glu-Xaa-Ile-Val-Pro (SEQ ID NO: 120);

(g) Asp-Pro-Glu-Pro-Ala-Pro-Pro-Val-Pro-Thr-Thr-Ala-Ala-Ser-Pro-Pro-Ser (SEQ ID NO: 121);

(h) Ala-Pro-Lys-Thr-Tyr-Xaa-Glu-Glu-Leu-Lys-Gly-Thr-Asp-Thr-Gly (SEQ ID NO: 122);

(i) Asp-Pro-Ala-Ser-Ala-Pro-Asp-Val-Pro-Thr-Ala-Ala-Gln-Leu-Thr-Ser-Leu-Leu-Asn-Ser-Leu-Ala-Asp-Pro-Asn-Val-Ser-Phe-Ala-Asn (SEQ ID NO: 123);

(j) Xaa-Asp-Ser-Glu-Lys-Ser-Ala-Thr-Ile-Lys-Val-Thr-Asp-Ala-Ser; (SEQ ID NO: 129)

(k) Ala-Gly-Asp-Thr-Xaa-Ile-Tyr-Ile-Val-Gly-Asn-Leu-Thr-Ala-Asp; (SEQ ID NO: 130) or

(l) Ala-Pro-Glu-Ser-Gly-Ala-Gly-Leu-Gly-Gly-Thr-Val-Gln-Ala-Gly; (SEQ ID NO: 131)

wherein Xaa may be any amino acid.

In a related aspect, polypeptides are provided comprising an immunogenic portion of an *M. tuberculosis* antigen, or a variant of such an antigen that differs only in conservative substitutions and/or modifications, the antigen having one of the following N-terminal sequences:

(m) Xaa-Tyr-Ile-Ala-Tyr-Xaa-Thr-Thr-Ala-Gly-Ile-Val-Pro-Gly-Lys-Ile-Asn-Val-His-Leu-Val; (SEQ ID NO: 132) or

(n) Asp-Pro-Pro-Asp-Pro-His-Gln-Xaa-Asp-Met-Thr-Lys-Gly-Tyr-Tyr-Pro-Gly-Gly-Arg-Arg-Xaa-Phe; (SEQ ID NO: 124)

wherein Xaa may be any amino acid.

In another embodiment, the soluble *M. tuberculosis* antigen comprises an amino acid sequence encoded by a DNA sequence selected from the group consisting of the sequences recited in SEQ ID NOS: 1, 2, 4-10, 13-25, 52, 94 and 96, the complements of said sequences, and DNA sequences that hybridize to a sequence recited in SEQ ID NOS: 1, 2, 4-10, 13-25, 52, 94 and 96 or a complement thereof under moderately stringent conditions.

In a related aspect, the polypeptides comprise an antigenic portion of a *M. tuberculosis* antigen, or a variant of such an antigen that differs only in conservative substitutions and/or modifications, wherein the antigen comprises an amino acid sequence encoded by a DNA sequence selected from the group consisting of the sequences recited in SEQ ID NOS. 26-51, 133, 134, 158-178 and 196, the complements of said sequences, and DNA sequences that hybridize to a sequence recited in SEQ ID NOS: 26-51, 133, 134, 158-178 and 196 or a complement thereof under moderately stringent conditions.

In related aspects, DNA sequences encoding the above polypeptides, recombinant expression vectors comprising these DNA sequences and host cells transformed or transfected with such expression vectors are also provided.

In another aspect, the present invention provides fusion proteins comprising a first and a second inventive polypeptide or, alternatively, an inventive polypeptide and a known *M. tuberculosis* antigen.

In further aspects of the subject invention, methods and diagnostic kits are provided for detecting tuberculosis in a patient. The methods comprise: (a) contacting a biological sample with at least one of the above polypeptides; and (b) detecting in the sample the presence of antibodies that bind to the polypeptide or polypeptides, thereby detecting *M. tuberculosis* infection in the biological sample. Suitable biological samples include whole blood, sputum, serum, plasma, saliva, cerebrospinal fluid and urine. The diagnostic kits comprise one or more of the above polypeptides in combination with a detection reagent.

The present invention also provides methods for detecting *M. tuberculosis* infection comprising: (a) obtaining a biological sample from a patient; (b) contacting the sample with at least one oligonucleotide primer in a polymerase chain reaction, the oligonucleotide primer being specific for a DNA sequence encoding the above polypeptides; and (c) detecting in the sample a DNA sequence that amplifies in the presence of the first and second oligonucleotide primers. In one embodiment, the oligonucleotide primer comprises at least about 10 contiguous nucleotides of such a DNA sequence.

In a further aspect, the present invention provides a method for detecting *M. tuberculosis* infection in a patient comprising: (a) obtaining a biological sample from the patient; (b) contacting the sample with an oligonucleotide probe specific for a DNA sequence encoding the above polypeptides; and (c) detecting in the sample a DNA sequence that hybridizes to the oligonucleotide probe. In one embodiment, the oligonucleotide probe comprises at least about 15 contiguous nucleotides of such a DNA sequence.

In yet another aspect, the present invention provides antibodies, both polyclonal and monoclonal, that bind to the polypeptides described above, as well as methods for their use in the detection of *M. tuberculosis* infection.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE IDENTIFIERS

Figure 1A and B illustrate the stimulation of proliferation and interferon-γ production in T cells derived from a first and a second *M. tuberculosis*-immune donor, respectively, by the 14 Kd, 20 Kd and 26 Kd antigens described in Example 1.

Figures 2A-D illustrate the reactivity of antisera raised against secretory *M. tuberculosis* proteins, the known *M. tuberculosis* antigen 85b and the inventive antigens Tb38-1 and TbH-9, respectively, with *M. tuberculosis* lysate (lane 2), *M. tuberculosis* secretory proteins (lane 3), recombinant Tb38-1 (lane 4), recombinant TbH-9 (lane 5) and recombinant 85b (lane 5).

Figure 3A illustrates the stimulation of proliferation in a TbH-9-specific T cell clone by secretory *M. tuberculosis* proteins, recombinant TbH-9 and a control antigen, TbRa11.

Figure 3B illustrates the stimulation of interferon-γ production in a TbH-9-specific T cell clone by secretory *M. tuberculosis* proteins, PPD and recombinant TbH-9.

Figure 4 illustrates the reactivity of two representative polypeptides with sera from *M. tuberculosis*-infected and uninfected individuals, as compared to the reactivity of bacterial lysate.

Figure 5 shows the reactivity of four representative polypeptides with sera from *M. tuberculosis*-infected and uninfected individuals, as compared to the reactivity of the 38 kD antigen.

Figure 6 shows the reactivity of recombinant 38 kD and TbRa11 antigens with sera from *M. tuberculosis* patients, PPD positive donors and normal donors.

Figure 7 shows the reactivity of the antigen TbRa2A with 38 kD negative sera.

Figure 8 shows the reactivity of the antigen of SEQ ID NO: 60 with sera from *M. tuberculosis* patients and normal donors.

Figure 9 illustrates the reactivity of the recombinant antigen TbH-29 (SEQ ID NO: 137) with sera from *M. tuberculosis* patients, PPD positive donors and normal donors as determined by indirect ELISA.

Figure 10 illustrates the reactivity of the recombinant antigen TbH-33 (SEQ ID NO: 140) with sera from *M. tuberculosis* patients and from normal donors, and with a pool of sera from *M. tuberculosis* patients, as determined both by direct and indirect ELISA

Figure 11 illustrates the reactivity of increasing concentrations of the recombinant antigen TbH-33 (SEQ ID NO: 140) with sera from M. tuberculosis patients and from normal donors as determined by ELISA.

SEQ. ID NO. 1 is the DNA sequence of TbRa1.

SEQ. ID NO. 2 is the DNA sequence of TbRa10.

SEQ. ID NO. 3 is the DNA sequence of TbRa11.

SEQ. ID NO. 4 is the DNA sequence of TbRa12.

SEQ. ID NO. 5 is the DNA sequence of TbRa13.

SEQ. ID NO. 6 is the DNA sequence of TbRa16.

SEQ. ID NO. 7 is the DNA sequence of TbRa17.

SEQ. ID NO. 8 is the DNA sequence of TbRa18.

SEQ. ID NO. 9 is the DNA sequence of TbRa19.

SEQ. ID NO. 10 is the DNA sequence of TbRa24.

SEQ. ID NO. 11 is the DNA sequence of TbRa26.

SEQ. ID NO. 12 is the DNA sequence of TbRa28.

SEQ. ID NO. 13 is the DNA sequence of TbRa29.

SEQ. ID NO. 14 is the DNA sequence of TbRa2A.

SEQ. ID NO. 15 is the DNA sequence of TbRa3.

SEQ. ID NO. 16 is the DNA sequence of TbRa32.

SEQ. ID NO. 17 is the DNA sequence of TbRa35.

SEQ. ID NO. 18 is the DNA sequence of TbRa36.

SEQ. ID NO. 19 is the DNA sequence of TbRa4.

SEQ. ID NO. 20 is the DNA sequence of TbRa9.

SEQ. ID NO. 21 is the DNA sequence of TbRaB.

SEQ. ID NO. 22 is the DNA sequence of TbRaC.

SEQ. ID NO. 23 is the DNA sequence of TbRaD.

SEQ. ID NO. 24 is the DNA sequence of YYWCPG.

SEQ. ID NO. 25 is the DNA sequence of AAMK.

SEQ. ID NO. 26 is the DNA sequence of TbL-23.

SEQ. ID NO. 27 is the DNA sequence of TbL-24.

SEQ. ID NO. 28 is the DNA sequence of TbL-25.

SEQ. ID NO. 29 is the DNA sequence of TbL-28.

SEQ. ID NO. 30 is the DNA sequence of TbL-29.

SEQ. ID NO. 31 is the DNA sequence of TbH-5.

SEQ. ID NO. 32 is the DNA sequence of TbH-8.

SEQ. ID NO. 33 is the DNA sequence of TbH-9.

SEQ. ID NO. 34 is the DNA sequence of TbM-1.

SEQ. ID NO. 35 is the DNA sequence of TbM-3.

SEQ. ID NO. 36 is the DNA sequence of TbM-6.

SEQ. ID NO. 37 is the DNA sequence of TbM-7.

SEQ. ID NO. 38 is the DNA sequence of TbM-9.

SEQ. ID NO. 39 is the DNA sequence of TbM-12.

SEQ. ID NO. 40 is the DNA sequence of TbM-13.

SEQ. ID NO. 41 is the DNA sequence of TbM-14.

SEQ. ID NO. 42 is the DNA sequence of TbM-15.

SEQ. ID NO. 43 is the DNA sequence of TbH-4.

SEQ. ID NO. 44 is the DNA sequence of TbH-4-FWD.

SEQ. ID NO. 45 is the DNA sequence of TbH-12.

SEQ. ID NO. 46 is the DNA sequence of Tb38-1.

SEQ. ID NO. 47 is the DNA sequence of Tb38-4.

SEQ. ID NO. 48 is the DNA sequence of TbL-17.

SEQ. ID NO. 49 is the DNA sequence of TbL-20.

SEQ. ID NO. 50 is the DNA sequence of TbL-21.

SEQ. ID NO. 51 is the DNA sequence of TbH-16.

SEQ. ID NO. 52 is the DNA sequence of DPEP.

SEQ. ID NO. 53 is the deduced amino acid sequence of DPEP.

SEQ. ID NO. 54 is the protein sequence of DPV N-terminal Antigen.

SEQ. ID NO. 55 is the protein sequence of AVGS N-terminal Antigen.

SEQ. ID NO. 56 is the protein sequence of AAMK N-terminal Antigen.

SEQ. ID NO. 57 is the protein sequence of YYWC N-terminal Antigen.

SEQ. ID NO. 58 is the protein sequence of DIGS N-terminal Antigen.

SEQ. ID NO. 59 is the protein sequence of AEES N-terminal Antigen.

SEQ. ID NO. 60 is the protein sequence of DPEP N-terminal Antigen.

SEQ. ID NO. 61 is the protein sequence of APKT N-terminal Antigen.

SEQ. ID NO. 62 is the protein sequence of DPAS N-terminal Antigen.

SEQ. ID NO. 63 is the deduced amino acid sequence of TbM-1 Peptide.

SEQ. ID NO. 64 is the deduced amino acid sequence of TbRa1.

SEQ. ID NO. 65 is the deduced amino acid sequence of TbRa10.

SEQ. ID NO. 66 is the deduced amino acid sequence of TbRa11.

SEQ. ID NO. 67 is the deduced amino acid sequence of TbRa12.

SEQ. ID NO. 68 is the deduced amino acid sequence of TbRa13.

SEQ. ID NO. 69 is the deduced amino acid sequence of TbRa16.

SEQ. ID NO. 70 is the deduced amino acid sequence of TbRa17.

SEQ. ID NO. 71 is the deduced amino acid sequence of TbRa18.

SEQ. ID NO. 72 is the deduced amino acid sequence of TbRa19.

SEQ. ID NO. 73 is the deduced amino acid sequence of TbRa24.

SEQ. ID NO. 74 is the deduced amino acid sequence of TbRa26.

SEQ. ID NO. 75 is the deduced amino acid sequence of TbRa28.

SEQ. ID NO. 76 is the deduced amino acid sequence of TbRa29.

SEQ. ID NO. 77 is the deduced amino acid sequence of TbRa2A.

SEQ. ID NO. 78 is the deduced amino acid sequence of TbRa3.

SEQ. ID NO. 79 is the deduced amino acid sequence of TbRa32.

SEQ. ID NO. 80 is the deduced amino acid sequence of TbRa35.

SEQ. ID NO. 81 is the deduced amino acid sequence of TbRa36.

SEQ. ID NO. 82 is the deduced amino acid sequence of TbRa4.

SEQ. ID NO. 83 is the deduced amino acid sequence of TbRa9.

SEQ. ID NO. 84 is the deduced amino acid sequence of TbRaB.

SEQ. ID NO. 85 is the deduced amino acid sequence of TbRaC.

SEQ. ID NO. 86 is the deduced amino acid sequence of TbRaD.

SEQ. ID NO. 87 is the deduced amino acid sequence of YYWCPG.

SEQ. ID NO. 88 is the deduced amino acid sequence of TbAAMK.

SEQ. ID NO. 89 is the deduced amino acid sequence of Tb38-1.

SEQ. ID NO. 90 is the deduced amino acid sequence of TbH-4.

SEQ. ID NO. 91 is the deduced amino acid sequence of TbH-8.

SEQ. ID NO. 92 is the deduced amino acid sequence of TbH-9.

SEQ. ID NO. 93 is the deduced amino acid sequence of TbH-12.

SEQ. ID NO. 94 is the DNA sequence of DPAS.

SEQ. ID NO. 95 is the deduced amino acid sequence of DPAS.

SEQ. ID NO. 96 is the DNA sequence of DPV.

SEQ. ID NO. 97 is the deduced amino acid sequence of DPV.

SEQ. ID NO. 98 is the DNA sequence of ESAT-6.

SEQ. ID NO. 99 is the deduced amino acid sequence of ESAT-6.

SEQ. ID NO. 100 is the DNA sequence of TbH-8-2.

SEQ. ID NO. 101 is the DNA sequence of TbH-9FL.

SEQ. ID NO. 102 is the deduced amino acid sequence of TbH-9FL.

SEQ. ID NO. 103 is the DNA sequence of TbH-9-1.

SEQ. ID NO. 104 is the deduced amino acid sequence of TbH-9-1.

SEQ. ID NO. 105 is the DNA sequence of TbH-9-4.

SEQ. ID NO. 106 is the deduced amino acid sequence of TbH-9-4.

SEQ. ID NO. 107 is the DNA sequence of Tb38-1F2 IN.

SEQ. ID NO. 108 is the DNA sequence of Tb38-1F2 RP.

SEQ. ID NO. 109 is the deduced amino acid sequence of Tb37-FL.

SEQ. ID NO. 110 is the deduced amino acid sequence of Tb38-IN.

SEQ. ID NO. 111 is the DNA sequence of Tb38-1F3.

SEQ. ID NO. 112 is the deduced amino acid sequence of Tb38-1F3.

SEQ. ID NO. 113 is the DNA sequence of Tb38-1F5.

SEQ. ID NO. 114 is the DNA sequence of Tb38-1F6.

SEQ. ID NO. 115 is the deduced N-terminal amino acid sequence of DPV.

SEQ. ID NO. 116 is the deduced N-terminal amino acid sequence of AVGS.

SEQ. ID NO. 117 is the deduced N-terminal amino acid sequence of AAMK.

SEQ. ID NO. 118 is the deduced N-terminal amino acid sequence of YYWC.

SEQ. ID NO. 119 is the deduced N-terminal amino acid sequence of DIGS.

SEQ. ID NO. 120 is the deduced N-terminal amino acid sequence of AAES.

SEQ. ID NO. 121 is the deduced N-terminal amino acid sequence of DPEP.

SEQ. ID NO. 122 is the deduced N-terminal amino acid sequence of APKT.

SEQ. ID NO. 123 is the deduced N-terminal amino acid sequence of DPAS.

SEQ. ID NO. 124 is the protein sequence of DPPD N-terminal Antigen.

SEQ ID NO. 125-128 are the protein sequences of four DPPD cyanogen bromide fragments.

SEQ ID NO. 129 is the N-terminal protein sequence of XDS antigen.

SEQ ID NO. 130 is the N-terminal protein sequence of AGD antigen.

SEQ ID NO. 131 is the N-terminal protein sequence of APE antigen.

SEQ ID NO. 132 is the N-terminal protein sequence of XYI antigen.

SEQ ID NO. 133 is the DNA sequence of TbH-29.

SEQ ID NO. 134 is the DNA sequence of TbH-30.

SEQ ID NO. 135 is the DNA sequence of TbH-32.

SEQ ID NO. 136 is the DNA sequence of TbH-33.

SEQ ID NO. 137 is the predicted amino acid sequence of TbH-29.

SEQ ID NO. 138 is the predicted amino acid sequence of TbH-30.

SEQ ID NO. 139 is the predicted amino acid sequence of TbH-32.

SEQ ID NO. 140 is the predicted amino acid sequence of TbH-33.

SEQ ID NO: 141-146 are PCR primers used in the preparation of a fusion protein containing TbRa3, 38 kD and Tb38-1.

SEQ ID NO: 147 is the DNA sequence of the fusion protein containing TbRa3, 38 kD and Tb38-1.

SEQ ID NO: 148 is the amino acid sequence of the fusion protein containing TbRa3, 38 kD and Tb38-1.

SEQ ID NO: 149 is the DNA sequence of the M. tuberculosis antigen 38 kD.

SEQ ID NO: 150 is the amino acid sequence of the M. tuberculosis antigen 38 kD.

SEQ ID NO: 151 is the DNA sequence of XP14.

SEQ ID NO: 152 is the DNA sequence of XP24.

SEQ ID NO: 153 is the DNA sequence of XP31.

SEQ ID NO: 154 is the 5` DNA sequence of XP32.

SEQ ID NO: 155 is the 3` DNA sequence of XP32.

SEQ ID NO: 156 is the predicted amino acid sequence of XP14.

SEQ ID NO: 157 is the predicted amino acid sequence encoded by the reverse complement of XP14.

SEQ ID NO: 158 is the DNA sequence of XP27.

SEQ ID NO: 159 is the DNA sequence of XP36.

SEQ ID NO: 160 is the 5' DNA sequence of XP4.

SEQ ID NO: 161 is the 5' DNA sequence of XP5.

SEQ ID NO: 162 is the 5' DNA sequence of XP17.

SEQ ID NO: 163 is the 5' DNA sequence of XP30.

SEQ ID NO: 164 is the 5' DNA sequence of XP2.

SEQ ID NO: 165 is the 3' DNA sequence of XP2.

SEQ ID NO: 166 is the 5' DNA sequence of XP3.

SEQ ID NO: 167 is the 3' DNA sequence of XP3.

SEQ ID NO: 168 is the 5' DNA sequence of XP6.

SEQ ID NO: 169 is the 3' DNA sequence of XP6.

SEQ ID NO: 170 is the 5' DNA sequence of XP18.

SEQ ID NO: 171 is the 3' DNA sequence of XP18.

SEQ ID NO: 172 is the 5' DNA sequence of XP19.

SEQ ID NO: 173 is the 3' DNA sequence of XP19.

SEQ ID NO: 174 is the 5' DNA sequence of XP22.

SEQ ID NO: 175 is the 3' DNA sequence of XP22.

SEQ ID NO: 176 is the 5' DNA sequence of XP25.

SEQ ID NO: 177 is the 3' DNA sequence of XP25.

SEQ ID NO: 178 is the full-length DNA sequence of TbH4-XP1.

SEQ ID NO: 179 is the predicted amino acid sequence of TbH4-XP1.

SEQ ID NO: 180 is the predicted amino acid sequence encoded by the reverse complement of TbH4-XP1.

SEQ ID NO: 181 is a first predicted amino acid sequence encoded by XP36.

SEQ ID NO: 182 is a second predicted amino acid sequence encoded by XP36.

SEQ ID NO: 183 is the predicted amino acid sequence encoded by the reverse complement of XP36.

SEQ ID NO: 184 is the DNA sequence of RDIF2.

SEQ ID NO: 185 is the DNA sequence of RDIF5.

SEQ ID NO: 186 is the DNA sequence of RDIF8.

SEQ ID NO: 187 is the DNA sequence of RDIF10.

SEQ ID NO: 188 is the DNA sequence of RDIF11.

SEQ ID NO: 189 is the predicted amino acid sequence of RDIF2.

SEQ ID NO: 190 is the predicted amino acid sequence of RDIF5.

SEQ ID NO: 191 is the predicted amino acid sequence of RDIF8.

SEQ ID NO: 192 is the predicted amino acid sequence of RDIF10.

SEQ ID NO: 193 is the predicted amino acid sequence of RDIF11.

SEQ ID NO: 194 is the 5' DNA sequence of RDIF12.

SEQ ID NO: 195 is the 3' DNA sequence of RDIF12.

SEQ ID NO: 196 is the DNA sequence of RDIF7.

SEQ ID NO: 197 is the predicted amino acid sequence of RDIF7.

SEQ ID NO: 198 is the DNA sequence of DIF2-1.

SEQ ID NO: 199 is the predicted amino acid sequence of DIF2-1.

SEQ ID NO: 200-207 are PCR primers used in the preparation of a fusion protein containing TbRa3, 38 kD, Tb38-1 and DPEP (hereinafter referred to as TbF-2).

SEQ ID NO: 208 is the DNA sequence of the fusion protein TbF-2.

SEQ ID NO: 209 is the amino acid sequence of the fusion protein TbF-2.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to compositions and methods for diagnosing tuberculosis. The compositions of the subject invention include polypeptides that comprise at least one antigenic portion of a *M. tuberculosis* antigen, or a variant of such an antigen that differs only in conservative substitutions and/or modifications. Polypeptides within the scope of the present invention include, but are not limited to, soluble *M. tuberculosis* antigens. A "soluble *M. tuberculosis* antigen" is a protein of *M. tuberculosis* origin that is present in *M. tuberculosis* culture filtrate. As used herein, the term "polypeptide" encompasses amino acid chains of any length, including full length proteins (*i.e.*, antigens), wherein the amino acid residues are linked by covalent peptide bonds. Thus,

a polypeptide comprising an antigenic portion of one of the above antigens may consist entirely of the antigenic portion, or may contain additional sequences. The additional sequences may be derived from the native *M. tuberculosis* antigen or may be heterologous, and such sequences may (but need not) be antigenic.

An "antigenic portion" of an antigen (which may or may not be soluble) is a portion that is capable of reacting with sera obtained from an *M. tuberculosis*-infected individual (*i.e.*, generates an absorbance reading with sera from infected individuals that is at least three standard deviations above the absorbance obtained with sera from uninfected individuals, in a representative ELISA assay described herein). An "*M. tuberculosis*-infected individual" is a human who has been infected with *M. tuberculosis* (*e.g.*, has an intradermal skin test response to PPD that is at least 0.5 cm in diameter). Infected individuals may display symptoms of tuberculosis or may be free of disease symptoms. Polypeptides comprising at least an antigenic portion of one or more *M. tuberculosis* antigens as described herein may generally be used, alone or in combination, to detect tuberculosis in a patient.

The compositions and methods of this invention also encompass variants of the above polypeptides. A "variant," as used herein, is a polypeptide that differs from the native antigen only in conservative substitutions and/or modifications, such that the antigenic properties of the polypeptide are retained. Such variants may generally be identified by modifying one of the above polypeptide sequences, and evaluating the antigenic properties of the modified polypeptide using, for example, the representative procedures described herein.

A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. In general, the following groups of amino acids represent conservative changes: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his.

Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the antigenic properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-

translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (*e.g.*, poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

In a related aspect, combination polypeptides are disclosed. A "combination polypeptide" is a polypeptide comprising at least one of the above antigenic portions and one or more additional antigenic *M. tuberculosis* sequences, which are joined via a peptide linkage into a single amino acid chain. The sequences may be joined directly (*i.e.*, with no intervening amino acids) or may be joined by way of a linker sequence (*e.g.*, Gly-Cys-Gly) that does not significantly diminish the antigenic properties of the component polypeptides.

In general, *M. tuberculosis* antigens, and DNA sequences encoding such antigens, may be prepared using any of a variety of procedures. For example, soluble antigens may be isolated from *M. tuberculosis* culture filtrate by procedures known to those of ordinary skill in the art, including anion-exchange and reverse phase chromatography. Purified antigens may then be evaluated for a desired property, such as the ability to react with sera obtained from an *M. tuberculosis*-infected individual. Such screens may be performed using the representative methods described herein. Antigens may then be partially sequenced using, for example, traditional Edman chemistry. *See* Edman and Berg, *Eur. J. Biochem. 80*:116-132, 1967.

Antigens may also be produced recombinantly using a DNA sequence that encodes the antigen, which has been inserted into an expression vector and expressed in an appropriate host. DNA molecules encoding soluble antigens may be isolated by screening an appropriate *M. tuberculosis* expression library with anti-sera (*e.g.*, rabbit) raised specifically against soluble *M. tuberculosis* antigens. DNA sequences encoding antigens that may or may not be soluble may be identified by screening an appropriate *M. tuberculosis* genomic or cDNA expression library with sera obtained from patients infected with *M. tuberculosis*. Such screens may generally be performed using techniques well known in the art, such as those described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989.

DNA sequences encoding soluble antigens may also be obtained by screening an appropriate *M. tuberculosis* cDNA or genomic DNA library for DNA sequences that hybridize to degenerate oligonucleotides derived from partial amino acid sequences of isolated soluble antigens. Degenerate oligonucleotide sequences for use in such a screen may be designed and synthesized, and the screen may be performed, as described (for example) in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (and references cited therein). Polymerase chain reaction (PCR) may also be employed, using the above oligonucleotides in methods well known in the art, to isolate a nucleic acid probe from a cDNA or genomic library. The library screen may then be performed using the isolated probe.

Regardless of the method of preparation, the antigens described herein are "antigenic." More specifically, the antigens have the ability to react with sera obtained from an *M. tuberculosis*-infected individual. Reactivity may be evaluated using, for example, the representative ELISA assays described herein, where an absorbance reading with sera from infected individuals that is at least three standard deviations above the absorbance obtained with sera from uninfected individuals is considered positive.

Antigenic portions of *M. tuberculosis* antigens may be prepared and identified using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3d ed., Raven Press, 1993, pp. 243-247 and references cited therein. Such techniques include screening polypeptide portions of the native antigen for antigenic properties. The representative ELISAs described herein may generally be employed in these screens. An antigenic portion of a polypeptide is a portion that, within such representative assays, generates a signal in such assays that is substantially similar to that generated by the full length antigen. In other words, an antigenic portion of a *M. tuberculosis* antigen generates at least about 20%, and preferably about 100%, of the signal induced by the full length antigen in a model ELISA as described herein.

Portions and other variants of *M. tuberculosis* antigens may be generated by synthetic or recombinant means. Synthetic polypeptides having fewer than about 100 amino acids, and generally fewer than about 50 amino acids, may be generated using techniques well known in the art. For example, such polypeptides may be synthesized using any of the

commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. *See* Merrifield, *J. Am. Chem. Soc.* 85:2149-2146, 1963. Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Applied BioSystems, Inc., Foster City, CA, and may be operated according to the manufacturer's instructions. Variants of a native antigen may generally be prepared using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis. Sections of the DNA sequence may also be removed using standard techniques to permit preparation of truncated polypeptides.

Recombinant polypeptides containing portions and/or variants of a native antigen may be readily prepared from a DNA sequence encoding the polypeptide using a variety of techniques well known to those of ordinary skill in the art. For example, supernatants from suitable host/vector systems which secrete recombinant protein into culture media may be first concentrated using a commercially available filter. Following concentration, the concentrate may be applied to a suitable purification matrix such as an affinity matrix or an ion exchange resin. Finally, one or more reverse phase HPLC steps can be employed to further purify a recombinant protein.

Any of a variety of expression vectors known to those of ordinary skill in the art may be employed to express recombinant polypeptides as described herein. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher eukaryotic cells. Preferably, the host cells employed are *E. coli*, yeast or a mammalian cell line, such as COS or CHO. The DNA sequences expressed in this manner may encode naturally occurring antigens, portions of naturally occurring antigens, or other variants thereof.

In general, regardless of the method of preparation, the polypeptides disclosed herein are prepared in substantially pure form. Preferably, the polypeptides are at least about 80% pure, more preferably at least about 90% pure and most preferably at least about 99% pure. For use in the methods described herein, however, such substantially pure polypeptides may be combined.

In certain specific embodiments, the subject invention discloses polypeptides comprising at least an antigenic portion of a soluble *M. tuberculosis* antigen (or a variant of such an antigen), where the antigen has one of the following N-terminal sequences:

(a) Asp-Pro-Val-Asp-Ala-Val-Ile-Asn-Thr-Thr-Cys-Asn-Tyr-Gly-Gln-Val-Val-Ala-Ala-Leu (SEQ ID NO: 115);

(b) Ala-Val-Glu-Ser-Gly-Met-Leu-Ala-Leu-Gly-Thr-Pro-Ala-Pro-Ser (SEQ ID NO: 116);

(c) Ala-Ala-Met-Lys-Pro-Arg-Thr-Gly-Asp-Gly-Pro-Leu-Glu-Ala-Ala-Lys-Glu-Gly-Arg (SEQ ID NO: 117);

(d) Tyr-Tyr-Trp-Cys-Pro-Gly-Gln-Pro-Phe-Asp-Pro-Ala-Trp-Gly-Pro (SEQ ID NO: 118);

(e) Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Gln-Xaa-Ala-Val (SEQ ID NO: 119);

(f) Ala-Glu-Glu-Ser-Ile-Ser-Thr-Xaa-Glu-Xaa-Ile-Val-Pro (SEQ ID NO: 120);

(g) Asp-Pro-Glu-Pro-Ala-Pro-Pro-Val-Pro-Thr-Thr-Ala-Ala-Ser-Pro-Pro-Ser (SEQ ID NO: 121);

(h) Ala-Pro-Lys-Thr-Tyr-Xaa-Glu-Glu-Leu-Lys-Gly-Thr-Asp-Thr-Gly (SEQ ID NO: 122);

(i) Asp-Pro-Ala-Ser-Ala-Pro-Asp-Val-Pro-Thr-Ala-Ala-Gln-Gln-Thr-Ser-Leu-Leu-Asn-Ser-Leu-Ala-Asp-Pro-Asn-Val-Ser-Phe-Ala-Asn (SEQ ID NO: 123);

(j) Xaa-Asp-Ser-Glu-Lys-Ser-Ala-Thr-Ile-Lys-Val-Thr-Asp-Ala-Ser; (SEQ ID NO: 129)

(k) Ala-Gly-Asp-Thr-Xaa-Ile-Tyr-Ile-Val-Gly-Asn-Leu-Thr-Ala-Asp; (SEQ ID NO: 130) or

(l) Ala-Pro-Glu-Ser-Gly-Ala-Gly-Leu-Gly-Gly-Thr-Val-Gln-Ala-Gly; (SEQ ID NO: 131)

wherein Xaa may be any amino acid, preferably a cysteine residue. A DNA sequence encoding the antigen identified as (g) above is provided in SEQ ID NO: 52, the deduced

amino acid sequence of which is provided in SEQ ID NO: 53. A DNA sequence encoding the antigen identified as (a) above is provided in SEQ ID NO: 96; its deduced amino acid sequence is provided in SEQ ID NO: 97. A DNA sequence corresponding to antigen (d) above is provided in SEQ ID NO: 24, a DNA sequence corresponding to antigen (c) is provided in SEQ ID NO: 25 and a DNA sequence corresponding to antigen (f) is disclosed in SEQ ID NO: 94 and its deduced amino acid sequence is provided in SEQ ID NO: 95.

In a further specific embodiment, the subject invention discloses polypeptides comprising at least an immunogenic portion of an *M. tuberculosis* antigen having one of the following N-terminal sequences, or a variant thereof that differs only in conservative substitutions and/or modifications:

(m) Xaa-Tyr-Ile-Ala-Tyr-Xaa-Thr-Thr-Ala-Gly-Ile-Val-Pro-Gly-Lys-Ile-Asn-Val-His-Leu-Val; (SEQ ID NO: 132) or

(n) Asp-Pro-Pro-Asp-Pro-His-Gln-Xaa-Asp-Met-Thr-Lys-Gly-Tyr-Tyr-Pro-Gly-Gly-Arg-Arg-Xaa-Phe; (SEQ ID NO: 124)

wherein Xaa may be any amino acid, preferably a cysteine residue.

In other specific embodiments, the subject invention discloses polypeptides comprising at least an antigenic portion of a soluble *M. tuberculosis* antigen (or a variant of such an antigen) that comprises one or more of the amino acid sequences encoded by (a) the DNA sequences of SEQ ID NOS: 1, 2, 4-10, 13-25, 52, 94 and 96, (b) the complements of such DNA sequences, or (c) DNA sequences substantially homologous to a sequence in (a) or (b).

In further specific embodiments, the subject invention discloses polypeptides comprising at least an antigenic portion of a *M. tuberculosis* antigen (or a variant of such an antigen), which may or may not be soluble, that comprises one or more of the amino acid sequences encoded by (a) the DNA sequences of SEQ ID NOS: 26-51, 133, 134, 158-178 and 196, (b) the complements of such DNA sequences or (c) DNA sequences substantially homologous to a sequence in (a) or (b).

In the specific embodiments discussed above, the *M. tuberculosis* antigens include variants that are encoded DNA sequences which are substantially homologous to one

or more of DNA sequences specifically recited herein. "Substantial homology," as used herein, refers to DNA sequences that are capable of hybridizing under moderately stringent conditions. Suitable moderately stringent conditions include prewashing in a solution of 5X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5X SSC, overnight or, in the event of cross-species homology, at 45°C with 0.5X SSC; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS). Such hybridizing DNA sequences are also within the scope of this invention, as are nucleotide sequences that, due to code degeneracy, encode an immunogenic polypeptide that is encoded by a hybridizing DNA sequence.

In a related aspect, the present invention provides fusion proteins comprising a first and a second inventive polypeptide or, alternatively, a polypeptide of the present invention and a known *M. tuberculosis* antigen, such as the 38 kD antigen described above or ESAT-6 (SEQ ID NOS: 98 and 99), together with variants of such fusion proteins. The fusion proteins of the present invention may also include a linker peptide between the first and second polypeptides.

A DNA sequence encoding a fusion protein of the present invention is constructed using known recombinant DNA techniques to assemble separate DNA sequences encoding the first and second polypeptides into an appropriate expression vector. The 3' end of a DNA sequence encoding the first polypeptide is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide so that the reading frames of the sequences are in phase to permit mRNA translation of the two DNA sequences into a single fusion protein that retains the biological activity of both the first and the second polypeptides.

A peptide linker sequence may be employed to separate the first and the second polypeptides by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion protein using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic

or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea et al., *Gene 40*:39-46, 1985; Murphy et al., *Proc. Natl. Acad. Sci. USA 83*:8258-8562, 1986; U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. The linker sequence may be from 1 to about 50 amino acids in length. Peptide linker sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric hindrance.

In another aspect, the present invention provides methods for using the polypeptides described above to diagnose tuberculosis. In this aspect, methods are provided for detecting *M. tuberculosis* infection in a biological sample, using one or more of the above polypeptides, alone or in combination. In embodiments in which multiple polypeptides are employed, polypeptides other than those specifically described herein, such as the 38 kD antigen described in Andersen and Hansen, *Infect. Immun. 57*:2481-2488, 1989, may be included. As used herein, a "biological sample" is any antibody-containing sample obtained from a patient. Preferably, the sample is whole blood, sputum, serum, plasma, saliva, cerebrospinal fluid or urine. More preferably, the sample is a blood, serum or plasma sample obtained from a patient or a blood supply. The polypeptide(s) are used in an assay, as described below, to determine the presence or absence of antibodies to the polypeptide(s) in the sample, relative to a predetermined cut-off value. The presence of such antibodies indicates previous sensitization to mycobacterial antigens which may be indicative of tuberculosis.

In embodiments in which more than one polypeptide is employed, the polypeptides used are preferably complementary (*i.e.*, one component polypeptide will tend to detect infection in samples where the infection would not be detected by another component polypeptide). Complementary polypeptides may generally be identified by using each polypeptide individually to evaluate serum samples obtained from a series of patients known to be infected with *M. tuberculosis*. After determining which samples test positive (as described below) with each polypeptide, combinations of two or more polypeptides may be

formulated that are capable of detecting infection in most, or all, of the samples tested. Such polypeptides are complementary. For example, approximately 25-30% of sera from tuberculosis-infected individuals are negative for antibodies to any single protein, such as the 38 kD antigen mentioned above. Complementary polypeptides may, therefore, be used in combination with the 38 kD antigen to improve sensitivity of a diagnostic test.

There are a variety of assay formats known to those of ordinary skill in the art for using one or more polypeptides to detect antibodies in a sample. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988, which is incorporated herein by reference. In a preferred embodiment, the assay involves the use of polypeptide immobilized on a solid support to bind to and remove the antibody from the sample. The bound antibody may then be detected using a detection reagent that contains a reporter group. Suitable detection reagents include antibodies that bind to the antibody/polypeptide complex and free polypeptide labeled with a reporter group (*e.g.*, in a semi-competitive assay). Alternatively, a competitive assay may be utilized, in which an antibody that binds to the polypeptide is labeled with a reporter group and allowed to bind to the immobilized antigen after incubation of the antigen with the sample. The extent to which components of the sample inhibit the binding of the labeled antibody to the polypeptide is indicative of the reactivity of the sample with the immobilized polypeptide.

The solid support may be any solid material known to those of ordinary skill in the art to which the antigen may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681.

The polypeptides may be bound to the solid support using a variety of techniques known to those of ordinary skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "bound" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the antigen and functional groups on the support or may be a linkage by way of a cross-linking agent). Binding by adsorption to a well in a microtiter plate or to a

membrane is preferred. In such cases, adsorption may be achieved by contacting the polypeptide, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour and 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of polypeptide ranging from about 10 ng to about 1 µg, and preferably about 100 ng, is sufficient to bind an adequate amount of antigen.

Covalent attachment of polypeptide to a solid support may generally be achieved by first reacting the support with a bifunctional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, on the polypeptide. For example, the polypeptide may be bound to supports having an appropriate polymer coating using benzoquinone or by condensation of an aldehyde group on the support with an amine and an active hydrogen on the polypeptide (*see*, e.g., Pierce Immunotechnology Catalog and Handbook, 1991, at A12-A13).

In certain embodiments, the assay is an enzyme linked immunosorbent assay (ELISA). This assay may be performed by first contacting a polypeptide antigen that has been immobilized on a solid support, commonly the well of a microtiter plate, with the sample, such that antibodies to the polypeptide within the sample are allowed to bind to the immobilized polypeptide. Unbound sample is then removed from the immobilized polypeptide and a detection reagent capable of binding to the immobilized antibody-polypeptide complex is added. The amount of detection reagent that remains bound to the solid support is then determined using a method appropriate for the specific detection reagent.

More specifically, once the polypeptide is immobilized on the support as described above, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20™ (Sigma Chemical Co., St. Louis, MO) may be employed. The immobilized polypeptide is then incubated with the sample, and antibody is allowed to bind to the antigen. The sample may be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an appropriate contact time (*i.e.*, incubation time) is that period of time that is sufficient to detect the presence of antibody within a *M. tuberculosis*-infected sample. Preferably, the contact time is sufficient to achieve a level

of binding that is at least 95% of that achieved at equilibrium between bound and unbound antibody. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20™. Detection reagent may then be added to the solid support. An appropriate detection reagent is any compound that binds to the immobilized antibody-polypeptide complex and that can be detected by any of a variety of means known to those in the art. Preferably, the detection reagent contains a binding agent (such as, for example, Protein A, Protein G, immunoglobulin, lectin or free antigen) conjugated to a reporter group. Preferred reporter groups include enzymes (such as horseradish peroxidase), substrates, cofactors, inhibitors, dyes, radionuclides, luminescent groups, fluorescent groups and biotin. The conjugation of binding agent to reporter group may be achieved using standard methods known to those of ordinary skill in the art. Common binding agents may also be purchased conjugated to a variety of reporter groups from many commercial sources (e.g., Zymed Laboratories, San Francisco, CA, and Pierce, Rockford, IL).

The detection reagent is then incubated with the immobilized antibody-polypeptide complex for an amount of time sufficient to detect the bound antibody. An appropriate amount of time may generally be determined from the manufacturer's instructions or by assaying the level of binding that occurs over a period of time. Unbound detection reagent is then removed and bound detection reagent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

To determine the presence or absence of anti-*M. tuberculosis* antibodies in the sample, the signal detected from the reporter group that remains bound to the solid support is generally compared to a signal that corresponds to a predetermined cut-off value. In one preferred embodiment, the cut-off value is the average mean signal obtained when the immobilized antigen is incubated with samples from an uninfected patient. In general, a sample generating a signal that is three standard deviations above the predetermined cut-off value is considered positive for tuberculosis. In an alternate preferred embodiment, the cut-off value is determined using a Receiver Operator Curve, according to the method of Sackett et al., *Clinical Epidemiology: A Basic Science for Clinical Medicine*, Little Brown and Co., 1985, pp. 106-107. Briefly, in this embodiment, the cut-off value may be determined from a plot of pairs of true positive rates (*i.e.*, sensitivity) and false positive rates (100%-specificity) that correspond to each possible cut-off value for the diagnostic test result. The cut-off value on the plot that is the closest to the upper left-hand corner (*i.e.*, the value that encloses the largest area) is the most accurate cut-off value, and a sample generating a signal that is higher than the cut-off value determined by this method may be considered positive. Alternatively, the cut-off value may be shifted to the left along the plot, to minimize the false positive rate, or to the right, to minimize the false negative rate. In general, a sample generating a signal that is higher than the cut-off value determined by this method is considered positive for tuberculosis.

In a related embodiment, the assay is performed in a rapid flow-through or strip test format, wherein the antigen is immobilized on a membrane, such as nitrocellulose. In the flow-through test, antibodies within the sample bind to the immobilized polypeptide as the sample passes through the membrane. A detection reagent (*e.g.*, protein A-colloidal gold) then binds to the antibody-polypeptide complex as the solution containing the detection reagent flows through the membrane. The detection of bound detection reagent may then be performed as described above. In the strip test format, one end of the membrane to which polypeptide is bound is immersed in a solution containing the sample. The sample migrates along the membrane through a region containing detection reagent and to the area of immobilized polypeptide. Concentration of detection reagent at the polypeptide indicates the presence of anti-*M. tuberculosis* antibodies in the sample. Typically, the concentration of

detection reagent at that site generates a pattern, such as a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the amount of polypeptide immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of antibodies that would be sufficient to generate a positive signal in an ELISA, as discussed above. Preferably, the amount of polypeptide immobilized on the membrane ranges from about 25 ng to about 1 μg, and more preferably from about 50 ng to about 500 ng. Such tests can typically be performed with a very small amount (*e.g.*, one drop) of patient serum or blood.

Of course, numerous other assay protocols exist that are suitable for use with the polypeptides of the present invention. The above descriptions are intended to be exemplary only.

In yet another aspect, the present invention provides antibodies to the inventive polypeptides. Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In one such technique, an immunogen comprising the antigenic polypeptide is initially injected into any of a wide variety of mammals (*e.g.*, mice, rats, rabbits, sheep and goats). In this step, the polypeptides of this invention may serve as the immunogen without modification. Alternatively, particularly for relatively short polypeptides, a superior immune response may be elicited if the polypeptide is joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. The immunogen is injected into the animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Monoclonal antibodies specific for the antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol* 6:511-519, 1976, and improvements thereto. Briefly, these methods involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example,

from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. The polypeptides of this invention may be used in the purification process in, for example, an affinity chromatography step.

Antibodies may be used in diagnostic tests to detect the presence of *M. tuberculosis* antigens using assays similar to those detailed above and other techniques well known to those of skill in the art, thereby providing a method for detecting *M. tuberculosis* infection in a patient.

Diagnostic reagents of the present invention may also comprise DNA sequences encoding one or more of the above polypeptides, or one or more portions thereof. For example, at least two oligonucleotide primers may be employed in a polymerase chain reaction (PCR) based assay to amplify *M. tuberculosis*-specific cDNA derived from a biological sample, wherein at least one of the oligonucleotide primers is specific for a DNA molecule encoding a polypeptide of the present invention. The presence of the amplified cDNA is then detected using techniques well known in the art, such as gel electrophoresis. Similarly, oligonucleotide probes specific for a DNA molecule encoding a polypeptide of the

present invention may be used in a hybridization assay to detect the presence of an inventive polypeptide in a biological sample.

As used herein, the term "oligonucleotide primer/probe specific for a DNA molecule" means an oligonucleotide sequence that has at least about 80%, preferably at least about 90% and more preferably at least about 95%, identity to the DNA molecule in question. Oligonucleotide primers and/or probes which may be usefully employed in the inventive diagnostic methods preferably have at least about 10-40 nucleotides. In a preferred embodiment, the oligonucleotide primers comprise at least about 10 contiguous nucleotides of a DNA molecule encoding one of the polypeptides disclosed herein. Preferably, oligonucleotide probes for use in the inventive diagnostic methods comprise at least about 15 contiguous oligonucleotides of a DNA molecule encoding one of the polypeptides disclosed herein. Techniques for both PCR based assays and hybridization assays are well known in the art (see, for example, Mullis *et al. Ibid*; Ehrlich, *Ibid*). Primers or probes may thus be used to detect *M. tuberculosis*-specific sequences in biological samples. DNA probes or primers comprising oligonucleotide sequences described above may be used alone, in combination with each other, or with previously identified sequences, such as the 38 kD antigen discussed above.

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

EXAMPLE 1

PURIFICATION AND CHARACTERIZATION OF POLYPEPTIDES FROM *M. TUBERCULOSIS* CULTURE FILTRATE

This example illustrates the preparation of *M tuberculosis* soluble polypeptides from culture filtrate. Unless otherwise noted, all percentages in the following example are weight per volume.

M. tuberculosis (either H37Ra, ATCC No. 25177, or H37Rv, ATCC No. 25618) was cultured in sterile GAS media at 37°C for fourteen days. The media was then vacuum filtered (leaving the bulk of the cells) through a 0.45 μ filter into a sterile 2.5 L bottle. The media was then filtered through a 0.2 μ filter into a sterile 4 L bottle. NaN_3 was then added to the culture filtrate to a concentration of 0.04%. The bottles were then placed in a 4°C cold room.

The culture filtrate was concentrated by placing the filtrate in a 12 L reservoir that had been autoclaved and feeding the filtrate into a 400 ml Amicon stir cell which had been rinsed with ethanol and contained a 10,000 kDa MWCO membrane. The pressure was maintained at 60 psi using nitrogen gas. This procedure reduced the 12 L volume to approximately 50 ml.

The culture filtrate was then dialyzed into 0.1% ammonium bicarbonate using a 8,000 kDa MWCO cellulose ester membrane, with two changes of ammonium bicarbonate solution. Protein concentration was then determined by a commercially available BCA assay (Pierce, Rockford, IL).

The dialyzed culture filtrate was then lyophilized, and the polypeptides resuspended in distilled water. The polypeptides were then dialyzed against 0.01 mM 1,3 bis[tris(hydroxymethyl)-methylamino]propane, pH 7.5 (Bis-Tris propane buffer), the initial conditions for anion exchange chromatography. Fractionation was performed using gel profusion chromatography on a POROS 146 II Q/M anion exchange column 4.6 mm x 100 mm (Perseptive BioSystems, Framingham, MA) equilibrated in 0.01 mM Bis-Tris propane buffer pH 7.5. Polypeptides were eluted with a linear 0-0.5 M NaCl gradient in the above buffer system. The column eluent was monitored at a wavelength of 220 nm.

The pools of polypeptides eluting from the ion exchange column were dialyzed against distilled water and lyophilized. The resulting material was dissolved in 0.1% trifluoroacetic acid (TFA) pH 1.9 in water, and the polypeptides were purified on a Delta-Pak C18 column (Waters, Milford, MA) 300 Angstrom pore size, 5 micron particle size (3.9 x 150 mm). The polypeptides were eluted from the column with a linear gradient from 0-60% dilution buffer (0.1% TFA in acetonitrile). The flow rate was 0.75 ml/minute and the HPLC eluent was monitored at 214 nm. Fractions containing the eluted polypeptides were collected

to maximize the purity of the individual samples. Approximately 200 purified polypeptides were obtained.

The purified polypeptides were then screened for the ability to induce T-cell proliferation in PBMC preparations. The PBMCs from donors known to be PPD skin test positive and whose T cells were shown to proliferate in response to PPD and crude soluble proteins from MTB were cultured in medium comprising RPMI 1640 supplemented with 10% pooled human serum and 50 μg/ml gentamicin. Purified polypeptides were added in duplicate at concentrations of 0.5 to 10 μg/mL. After six days of culture in 96-well round-bottom plates in a volume of 200 μl, 50 μl of medium was removed from each well for determination of IFN-γ levels, as described below. The plates were then pulsed with 1 μCi/well of tritiated thymidine for a further 18 hours, harvested and tritium uptake determined using a gas scintillation counter. Fractions that resulted in proliferation in both replicates three fold greater than the proliferation observed in cells cultured in medium alone were considered positive.

IFN-γ was measured using an enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with a mouse monoclonal antibody directed to human IFN-γ (Chemicon) in PBS for four hours at room temperature. Wells were then blocked with PBS containing 5% (W/V) non-fat dried milk for 1 hour at room temperature. The plates were then washed six times in PBS/0.2% TWEEN-20 and samples diluted 1:2 in culture medium in the ELISA plates were incubated overnight at room temperature. The plates were again washed and a polyclonal rabbit anti-human IFN-γ serum diluted 1:3000 in PBS/10% normal goat serum was added to each well. The plates were then incubated for two hours at room temperature, washed and horseradish peroxidase-coupled anti-rabbit IgG (Jackson Labs.) was added at a 1:2000 dilution in PBS/5% non-fat dried milk. After a further two hour incubation at room temperature, the plates were washed and TMB substrate added. The reaction was stopped after 20 min with 1 N sulfuric acid. Optical density was determined at 450 nm using 570 nm as a reference wavelength. Fractions that resulted in both replicates giving an OD two fold greater than the mean OD from cells cultured in medium alone, plus 3 standard deviations, were considered positive.

For sequencing, the polypeptides were individually dried onto Biobrene™ (Perkin Elmer/Applied BioSystems Division, Foster City, CA) treated glass fiber filters. The filters with polypeptide were loaded onto a Perkin Elmer/Applied BioSystems Division Procise 492 protein sequencer. The polypeptides were sequenced from the amino terminal and using traditional Edman chemistry. The amino acid sequence was determined for each polypeptide by comparing the retention time of the PTH amino acid derivative to the appropriate PTH derivative standards.

Using the procedure described above, antigens having the following N-terminal sequences were isolated:

(a) Asp-Pro-Val-Asp-Ala-Val-Ile-Asn-Thr-Thr-Xaa-Asn-Tyr-Gly-Gln-Val-Val-Ala-Ala-Leu (SEQ ID NO: 54);

(b) Ala-Val-Glu-Ser-Gly-Met-Leu-Ala-Leu-Gly-Thr-Pro-Ala-Pro-Ser (SEQ ID NO: 55);

(c) Ala-Ala-Met-Lys-Pro-Arg-Thr-Gly-Asp-Gly-Pro-Leu-Glu-Ala-Ala-Lys-Glu-Gly-Arg (SEQ ID NO: 56);

(d) Tyr-Tyr-Trp-Cys-Pro-Gly-Gln-Pro-Phe-Asp-Pro-Ala-Trp-Gly-Pro (SEQ ID NO: 57);

(e) Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Gln-Xaa-Ala-Val (SEQ ID NO: 58);

(f) Ala-Glu-Glu-Ser-Ile-Ser-Thr-Xaa-Glu-Xaa-Ile-Val-Pro (SEQ ID NO: 59);

(g) Asp-Pro-Glu-Pro-Ala-Pro-Pro-Val-Pro-Thr-Ala-Ala-Ala-Ala-Pro-Pro-Ala (SEQ ID NO: 60); and

(h) Ala-Pro-Lys-Thr-Tyr-Xaa-Glu-Glu-Leu-Lys-Gly-Thr-Asp-Thr-Gly (SEQ ID NO: 61);

wherein Xaa may be any amino acid.

An additional antigen was isolated employing a microbore HPLC purification step in addition to the procedure described above. Specifically, 20 µl of a fraction comprising a mixture of antigens from the chromatographic purification step previously described, was purified on an Aquapore C18 column (Perkin Elmer/Applied Biosystems Division, Foster

City, CA) with a 7 micron pore size, column size 1 mm x 100 mm, in a Perkin Elmer/Applied Biosystems Division Model 172 HPLC. Fractions were eluted from the column with a linear gradient of 1%/minute of acetonitrile (containing 0.05% TFA) in water (0.05% TFA) at a flow rate of 80 µl/minute. The eluent was monitored at 250 nm. The original fraction was separated into 4 major peaks plus other smaller components and a polypeptide was obtained which was shown to have a molecular weight of 12,054 Kd (by mass spectrometry) and the following N-terminal sequence:

(i) Asp-Pro-Ala-Ser-Ala-Pro-Asp-Val-Pro-Thr-Ala-Ala-Gln-Gln-Thr-Ser-Leu-Leu-Asn-Asn-Leu-Ala-Asp-Pro-Asp-Val-Ser-Phe-Ala-Asp (SEQ ID NO: 62).

This polypeptide was shown to induce proliferation and IFN-γ production in PBMC preparations using the assays described above.

Additional soluble antigens were isolated from *M. tuberculosis* culture filtrate as follows. *M. tuberculosis* culture filtrate was prepared as described above. Following dialysis against Bis-Tris propane buffer, at pH 5.5, fractionation was performed using anion exchange chromatography on a Poros QE column 4.6 x 100 mm (Perseptive Biosystems) equilibrated in Bis-Tris propane buffer pH 5.5. Polypeptides were eluted with a linear 0-1.5 M NaCl gradient in the above buffer system at a flow rate of 10 ml/min. The column eluent was monitored at a wavelength of 214 nm.

The fractions eluting from the ion exchange column were pooled and subjected to reverse phase chromatography using a Poros R2 column 4.6 x 100 mm (Perseptive Biosystems). Polypeptides were eluted from the column with a linear gradient from 0-100% acetonitrile (0.1% TFA) at a flow rate of 5 ml/min. The eluent was monitored at 214 nm.

Fractions containing the eluted polypeptides were lyophilized and resuspended in 80 µl of aqueous 0.1% TFA and further subjected to reverse phase chromatography on a Vydac C4 column 4.6 x 150 mm (Western Analytical, Temecula, CA) with a linear gradient of 0-100% acetonitrile (0.1% TFA) at a flow rate of 2 ml/min. Eluent was monitored at 214 nm.

The fraction with biological activity was separated into one major peak plus other smaller components. Western blot of this peak onto PVDF membrane revealed three major bands of molecular weights 14 Kd, 20 Kd and 26 Kd. These polypeptides were determined to have the following N-terminal sequences, respectively:

(j) Xaa-Asp-Ser-Glu-Lys-Ser-Ala-Thr-Ile-Lys-Val-Thr-Asp-Ala-Ser; (SEQ ID NO: 129)

(k) Ala-Gly-Asp-Thr-Xaa-Ile-Tyr-Ile-Val-Gly-Asn-Leu-Thr-Ala-Asp; (SEQ ID NO: 130) and

(l) Ala-Pro-Glu-Ser-Gly-Ala-Gly-Leu-Gly-Gly-Thr-Val-Gln-Ala-Gly; (SEQ ID NO: 131), wherein Xaa may be any amino acid.

Using the assays described above, these polypeptides were shown to induce proliferation and IFN-γ production in PBMC preparations. Figs. 1A and B show the results of such assays using PBMC preparations from a first and a second donor, respectively.

DNA sequences that encode the antigens designated as (a), (c), (d) and (g) above were obtained by screening a *M. tuberculosis* genomic library using ^{32}P end labeled degenerate oligonucleotides corresponding to the N-terminal sequence and containing *M. tuberculosis* codon bias. The screen performed using a probe corresponding to antigen (a) above identified a clone having the sequence provided in SEQ ID NO: 96. The polypeptide encoded by SEQ ID NO: 96 is provided in SEQ ID NO: 97. The screen performed using a probe corresponding to antigen (g) above identified a clone having the sequence provided in SEQ ID NO: 52. The polypeptide encoded by SEQ ID NO: 52 is provided in SEQ ID NO: 53. The screen performed using a probe corresponding to antigen (d) above identified a clone having the sequence provided in SEQ ID NO: 24, and the screen performed with a probe corresponding to antigen (c) identified a clone having the sequence provided in SEQ ID NO: 25.

The above amino acid sequences were compared to known amino acid sequences in the gene bank using the DNA STAR system. The database searched contains some 173,000 proteins and is a combination of the Swiss, PIR databases along with translated protein sequences (Version 87). No significant homologies to the amino acid sequences for antigens (a)-(h) and (l) were detected.

The amino acid sequence for antigen (i) was found to be homologous to a sequence from *M. leprae*. The full length *M. leprae* sequence was amplified from genomic DNA using the sequence obtained from GENBANK. This sequence was then used to screen an *M. tuberculosis* library and a full length copy of the *M. tuberculosis* homologue was obtained (SEQ ID NO: 94).

The amino acid sequence for antigen (j) was found to be homologous to a known *M. tuberculosis* protein translated from a DNA sequence. To the best of the inventors' knowledge, this protein has not been previously shown to possess T-cell stimulatory activity. The amino acid sequence for antigen (k) was found to be related to a sequence from *M. leprae*.

In the proliferation and IFN-γ assays described above, using three PPD positive donors, the results for representative antigens provided above are presented in Table 1:

TABLE 1

RESULTS OF PBMC PROLIFERATION AND IFN-γ ASSAYS

Sequence	Proliferation	IFN-γ
(a)	+	-
(c)	+++	+++
(d)	++	++
(g)	+++	+++
(h)	+++	+++

In Table 1, responses that gave a stimulation index (SI) of between 2 and 4 (compared to cells cultured in medium alone) were scored as +, as SI of 4-8 or 2-4 at a concentration of 1 μg or less was scored as ++ and an SI of greater than 8 was scored as +++. The antigen of sequence (i) was found to have a high SI (+++) for one donor and lower SI (++ and +) for the two other donors in both proliferation and IFN-γ assays. These results

indicate that these antigens are capable of inducing proliferation and/or interferon-γ production.

EXAMPLE 2

USE OF PATIENT SERA TO ISOLATE *M. TUBERCULOSIS* ANTIGENS

This example illustrates the isolation of antigens from *M. tuberculosis* lysate by screening with serum from *M. tuberculosis*-infected individuals.

Dessicated *M. tuberculosis* H37Ra (Difco Laboratories) was added to a 2% NP40 solution, and alternately homogenized and sonicated three times. The resulting suspension was centrifuged at 13,000 rpm in microfuge tubes and the supernatant put through a 0.2 micron syringe filter. The filtrate was bound to Macro Prep DEAE beads (BioRad, Hercules, CA). The beads were extensively washed with 20 mM Tris pH 7.5 and bound proteins eluted with 1M NaCl. The NaCl elute was dialyzed overnight against 10 mM Tris, pH 7.5. Dialyzed solution was treated with DNase and RNase at 0.05 mg/ml for 30 min. at room temperature and then with α-D-mannosidase, 0.5 U/mg at pH 4.5 for 3-4 hours at room temperature. After returning to pH 7.5, the material was fractionated via FPLC over a Bio Scale-Q-20 column (BioRad). Fractions were combined into nine pools, concentrated in a Centriprep 10 (Amicon, Beverley, MA) and screened by Western blot for serological activity using a serum pool from *M. tuberculosis*-infected patients which was not immunoreactive with other antigens of the present invention.

The most reactive fraction was run in SDS-PAGE and transferred to PVDF. A band at approximately 85 Kd was cut out yielding the sequence:

(m) Xaa-Tyr-Ile-Ala-Tyr-Xaa-Thr-Thr-Ala-Gly-Ile-Val-Pro-Gly-Lys-Ile-Asn-Val-His-Leu-Val; (SEQ ID NO: 132), wherein Xaa may be any amino acid.

Comparison of this sequence with those in the gene bank as described above, revealed no significant homologies to known sequences.

A DNA sequence that encodes the antigen designated as (m) above was obtained by screening a genomic *M. tuberculosis* Erdman strain library using labeled

degenerate oligonucleotides corresponding to the N-terminal sequence of SEQ ID NO:137. A clone was identified having the DNA sequence provided in SEQ ID NO: 198. This sequence was found to encode the amino acid sequence provided in SEQ ID NO: 199. Comparison of these sequences with those in the genebank revealed some similarity to sequences previously identified in *M. tuberculosis* and *M. bovis*.

EXAMPLE 3

PREPARATION OF DNA SEQUENCES ENCODING *M. TUBERCULOSIS* ANTIGENS

This example illustrates the preparation of DNA sequences encoding *M. tuberculosis* antigens by screening a *M. tuberculosis* expression library with sera obtained from patients infected with *M. tuberculosis*, or with anti-sera raised against *M. tuberculosis* antigens.

A. PREPARATION OF *M. TUBERCULOSIS* SOLUBLE ANTIGENS USING RABBIT ANTI-SERA RAISED AGAINST M. TUBERCULOSIS SUPERNATANT

Genomic DNA was isolated from the *M. tuberculosis* strain H37Ra. The DNA was randomly sheared and used to construct an expression library using the Lambda ZAP expression system (Stratagene, La Jolla, CA). Rabbit anti-sera was generated against secretory proteins of the *M. tuberculosis* strains H37Ra, H37Rv and Erdman by immunizing a rabbit with concentrated supernatant of the *M. tuberculosis* cultures. Specifically, the rabbit was first immunized subcutaneously with 200 µg of protein antigen in a total volume of 2 ml containing 100 µg muramyl dipeptide (Calbiochem, La Jolla, CA) and 1 ml of incomplete Freund's adjuvant. Four weeks later the rabbit was boosted subcutaneously with 100 µg antigen in incomplete Freund's adjuvant. Finally, the rabbit was immunized intravenously four weeks later with 50 µg protein antigen. The anti-sera were used to screen the expression library as described in Sambrook et al., *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989. Bacteriophage plaques expressing immunoreactive antigens were purified. Phagemid from the plaques was rescued and the nucleotide sequences of the *M. tuberculosis* clones deduced.

Thirty two clones were purified. Of these, 25 represent sequences that have not been previously identified in *M. tuberculosis*. Proteins were induced by IPTG and purified by gel elution, as described in Skeiky et al., *J. Exp. Med. 181*:1527-1537, 1995. Representative partial sequences of DNA molecules identified in this screen are provided in SEQ ID NOS: 1-25. The corresponding predicted amino acid sequences are shown in SEQ ID NOS: 64-88.

On comparison of these sequences with known sequences in the gene bank using the databases described above, it was found that the clones referred to hereinafter as TbRA2A, TbRA16, TbRA18, and TbRA29 (SEQ ID NOS: 77, 69, 71, 76) show some homology to sequences previously identified in *Mycobacterium leprae* but not in *M. tuberculosis*. TbRA11, TbRA26, TbRA28 and TbDPEP (SEQ ID NOS: 66, 74, 75, 53) have been previously identified in *M. tuberculosis*. No significant homologies were found to TbRA1, TbRA3, TbRA4, TbRA9, TbRA10, TbRA13, TbRA17, TbRA19, TbRA29, TbRA32, TbRA36 and the overlapping clones TbRA35 and TbRA12 (SEQ ID NOS: 64, 78, 82, 83, 65, 68, 76, 72, 76, 79, 81, 80, 67, respectively). The clone TbRa24 is overlapping with clone TbRa29.

B. USE OF SERA FROM PATIENTS HAVING PULMONARY OR PLEURAL TUBERCULOSIS TO IDENTIFY DNA SEQUENCES ENCODING *M. TUBERCULOSIS* ANTIGENS

The genomic DNA library described above, and an additional H37Rv library, were screened using pools of sera obtained from patients with active tuberculosis. To prepare the H37Rv library, *M. tuberculosis* strain H37Rv genomic DNA was isolated, subjected to partial Sau3A digestion and used to construct an expression library using the Lambda Zap expression system (Stratagene, La Jolla, Ca). Three different pools of sera, each containing sera obtained from three individuals with active pulmonary or pleural disease, were used in the expression screening. The pools were designated TbL, TbM and TbH, referring to relative reactivity with H37Ra lysate (*i.e.*, TbL = low reactivity, TbM = medium reactivity and TbH = high reactivity) in both ELISA and immunoblot format. A fourth pool of sera from seven patients with active pulmonary tuberculosis was also employed. All of the sera

lacked increased reactivity with the recombinant 38 kD *M. tuberculosis* H37Ra phosphate-binding protein.

All pools were pre-adsorbed with *E. coli* lysate and used to screen the H37Ra and H37Rv expression libraries, as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989. Bacteriophage plaques expressing immunoreactive antigens were purified. Phagemid from the plaques was rescued and the nucleotide sequences of the *M. tuberculosis* clones deduced.

Thirty two clones were purified. Of these, 31 represented sequences that had not been previously identified in human *M. tuberculosis*. Representative sequences of the DNA molecules identified are provided in SEQ ID NOS:: 26-51 and 100. Of these, TbH-8-2 (SEQ. ID NO. 100) is a partial clone of TbH-8, and TbH-4 (SEQ. ID NO. 43) and TbH-4-FWD (SEQ. ID NO. 44) are non-contiguous sequences from the same clone. Amino acid sequences for the antigens hereinafter identified as Tb38-1, TbH-4, TbH-8, TbH-9, and TbH-12 are shown in SEQ ID NOS.: 89-93. Comparison of these sequences with known sequences in the gene bank using the databases identified above revealed no significant homologies to TbH-4, TbH-8, TbH-9 and TbM-3, although weak homologies were found to TbH-9. TbH-12 was found to be homologous to a 34 kD antigenic protein previously identified in *M. paratuberculosis* (Acc. No. S28515). Tb38-1 was found to be located 34 base pairs upstream of the open reading frame for the antigen ESAT-6 previously identified in *M bovis* (Acc. No. U34848) and in *M. tuberculosis* (Sorensen et al., *Infec. Immun. 63*:1710-1717, 1995).

Probes derived from Tb38-1 and TbH-9, both isolated from an H37Ra library, were used to identify clones in an H37Rv library. Tb38-1 hybridized to Tb38-1F2, Tb38-1F3, Tb38-1F5 and Tb38-1F6 (SEQ. ID NOS: 107, 108, 111, 113, and 114). (SEQ ID NOS: 107 and 108 are non-contiguous sequences from clone Tb38-1F2.) Two open reading frames were deduced in Tb38-1F2, one corresponds to Tb37FL (SEQ. ID. NO. 109), the second, a partial sequence, may be the homologue of Tb38-1 and is called Tb38-IN (SEQ. ID NO. 110). The deduced amino acid sequence of Tb38-1F3 is presented in SEQ. ID. NO. 112. A TbH-9 probe identified three clones in the H37Rv library: TbH-9-FL (SEQ. ID NO. 101), which may be the homologue of TbH-9 (R37Ra), TbH-9-1 (SEQ. ID NO. 103), and TbH-8-2 (SEQ.

ID NO. 105) is a partial clone of TbH-8. The deduced amino acid sequences for these three clones are presented in SEQ ID NOS: 102, 104 and 106.

Further screening of the *M. tuberculosis* genomic DNA library, as described above, resulted in the recovery of ten additional reactive clones, representing seven different genes. One of these genes was identified as the 38 Kd antigen discussed above, one was determined to be identical to the 14Kd alpha crystallin heat shock protein previously shown to be present in *M. tuberculosis*, and a third was determined to be identical to the antigen TbH-8 described above. The determined DNA sequences for the remaining five clones (hereinafter referred to as TbH-29, TbH-30, TbH-32 and TbH-33) are provided in SEQ ID NO: 133-136, respectively, with the corresponding predicted amino acid sequences being provided in SEQ ID NO: 137-140, respectively. The DNA and amino acid sequences for these antigens were compared with those in the gene bank as described above. No homologies were found to the 5' end of TbH-29 (which contains the reactive open reading frame), although the 3' end of TbH-29 was found to be identical to the *M. tuberculosis* cosmid Y227. TbH-32 and TbH-33 were found to be identical to the previously identified *M. tuberculosis* insertion element IS6110 and to the *M. tuberculosis* cosmid Y50, respectively. No significant homologies to TbH-30 were found.

Positive phagemid from this additional screening were used to infect *E. coli* XL-1 Blue MRF', as described in Sambrook et al., *supra*. Induction of recombinant protein was accomplished by the addition of IPTG. Induced and uninduced lysates were run in duplicate on SDS-PAGE and transferred to nitrocellulose filters. Filters were reacted with human *M. tuberculosis* sera (1:200 dilution) reactive with TbH and a rabbit sera (1:200 or 1:250 dilution) reactive with the N-terminal 4 Kd portion of lacZ. Sera incubations were performed for 2 hours at room temperature. Bound antibody was detected by addition of ^{125}I-labeled Protein A and subsequent exposure to film for variable times ranging from 16 hours to 11 days. The results of the immunoblots are summarized in Table 2.

TABLE 2

Antigen	Human M. tb Sera	Anti-lacZ Sera
TbH-29	45 Kd	45 Kd
TbH-30	No reactivity	29 Kd
TbH-32	12 Kd	12 Kd
TbH-33	16 Kd	16 Kd

Positive reaction of the recombinant human *M. tuberculosis* antigens with both the human *M. tuberculosis* sera and anti-lacZ sera indicate that reactivity of the human *M. tuberculosis* sera is directed towards the fusion protein. Antigens reactive with the anti-lacZ sera but not with the human *M. tuberculosis* sera may be the result of the human *M. tuberculosis* sera recognizing conformational epitopes, or the antigen-antibody binding kinetics may be such that the 2 hour sera exposure in the immunoblot is not sufficient.

Studies were undertaken to determine whether the antigens TbH-9 and Tb38-1 represent cellular proteins or are secreted into *M. tuberculosis* culture media. In the first study, rabbit sera were raised against A) secretory proteins of *M. tuberculosis*, B) the known secretory recombinant *M. tuberculosis* antigen 85b, C) recombinant Tb38-1 and D) recombinant TbH-9, using protocols substantially as described in Example 3A. Total *M. tuberculosis* lysate, concentrated supernatant of *M. tuberculosis* cultures and the recombinant antigens 85b, TbH-9 and Tb38-1 were resolved on denaturing gels, immobilized on nitrocellulose membranes and duplicate blots were probed using the rabbit sera described above.

The results of this analysis using control sera (panel I) and antisera (panel II) against secretory proteins, recombinant 85b, recombinant Tb38-1 and recombinant TbH-9 are shown in Figures 2A-D, respectively, wherein the lane designations are as follows: 1) molecular weight protein standards; 2) 5 µg of *M. tuberculosis* lysate; 3) 5 µg secretory proteins; 4) 50 ng recombinant Tb38-1; 5) 50 ng recombinant TbH-9; and 6) 50 ng recombinant 85b. The recombinant antigens were engineered with six terminal histidine

residues and would therefore be expected to migrate with a mobility approximately 1 kD larger that the native protein. In Figure 2D, recombinant TbH-9 is lacking approximately 10 kD of the full-length 42 kD antigen, hence the significant difference in the size of the immunoreactive native TbH-9 antigen in the lysate lane (indicated by an arrow). These results demonstrate that Tb38-1 and TbH-9 are intracellular antigens and are not actively secreted by *M. tuberculosis*.

The finding that TbH-9 is an intracellular antigen was confirmed by determining the reactivity of TbH-9-specific human T cell clones to recombinant TbH-9, secretory *M. tuberculosis* proteins and PPD. A TbH-9-specific T cell clone (designated 131TbH-9) was generated from PBMC of a healthy PPD-positive donor. The proliferative response of 131TbH-9 to secretory proteins, recombinant TbH-9 and a control *M. tuberculosis* antigen, TbRa11, was determined by measuring uptake of tritiated thymidine, as described in Example 1. As shown in Figure 3A, the clone 131TbH-9 responds specifically to TbH-9, showing that TbH-9 is not a significant component of *M. tuberculosis* secretory proteins. Figure 3B shows the production of IFN-γ by a second TbH-9-specific T cell clone (designated PPD 800-10) prepared from PBMC from a healthy PPD-positive donor, following stimulation of the T cell clone with secretory proteins, PPD or recombinant TbH-9. These results further confirm that TbH-9 is not secreted by *M. tuberculosis*.

C. USE OF SERA FROM PATIENTS HAVING EXTRAPULMONARY TUBERCULOSIS TO IDENTIFY DNA SEQUENCES ENCODING *M. TUBERCULOSIS* ANTIGENS

Genomic DNA was isolated from *M. tuberculosis* Erdman strain, randomly sheared and used to construct an expression library employing the Lambda ZAP expression system (Stratagene, La Jolla, CA). The resulting library was screened using pools of sera obtained from individuals with extrapulmonary tuberculosis, as described above in Example 3B, with the secondary antibody being goat anti-human IgG + A + M (H+L) conjugated with alkaline phosphatase.

Eighteen clones were purified. Of these, 4 clones (hereinafter referred to as XP14, XP24, XP31 and XP32) were found to bear some similarity to known sequences. The determined DNA sequences for XP14, XP24 and XP31 are provided in SEQ ID NOS: 151-

153, respectively, with the 5' and 3' DNA sequences for XP32 being provided in SEQ ID NOS: 154 and 155, respectively. The predicted amino acid sequence for XP14 is provided in SEQ ID NO: 156. The reverse complement of XP14 was found to encode the amino acid sequence provided in SEQ ID NO: 157.

Comparison of the sequences for the remaining 14 clones (hereinafter referred to as XP1-XP6, XP17-XP19, XP22, XP25, XP27, XP30 and XP36) with those in the genebank as described above, revealed no homologies with the exception of the 3' ends of XP2 and XP6 which were found to bear some homology to known *M. tuberculosis* cosmids. The DNA sequences for XP27 and XP36 are shown in SEQ ID NOS: 158 and 159, respectively, with the 5' sequences for XP4, XP5, XP17 and XP30 being shown in SEQ ID NOS: 160-163, respectively, and the 5' and 3' sequences for XP2, XP3, XP6, XP18, XP19, XP22 and XP25 being shown in SEQ ID NOS: 164 and 165; 166 and 167; 168 and 169; 170 and 171; 172 and 173; 174 and 175; and 176 and 177, respectively. XP1 was found to overlap with the DNA sequences for TbH4, disclosed above. The full-length DNA sequence for TbH4-XP1 is provided in SEQ ID NO: 178. This DNA sequence was found to contain an open reading frame encoding the amino acid sequence shown in SEQ ID NO: 179. The reverse complement of TbH4-XP1 was found to contain an open reading frame encoding the amino acid sequence shown in SEQ ID NO: 180. The DNA sequence for XP36 was found to contain two open reading frames encoding the amino acid sequence shown in SEQ ID NOS: 181 and 182, with the reverse complement containing an open reading frame encoding the amino acid sequence shown in SEQ ID NO: 183.

Recombinant XP1 protein was prepared as described above in Example 3B, with a metal ion affinity chromatography column being employed for purification. Recombinant XP1 was found to stimulate cell proliferation and IFN-γ production in T cells isolated from an *M. tuberculosis*-immune donors.

D. PREPARATION OF M. TUBERCULOSIS SOLUBLE ANTIGENS USING RABBIT ANTI-SERA RAISED AGAINST M. TUBERCULOSIS FRACTIONATED PROTEINS

M. tuberculosis lysate was prepared as described above in Example 2. The resulting material was fractionated by HPLC and the fractions screened by Western blot for

serological activity with a serum pool from *M. tuberculosis*-infected patients which showed little or no immunoreactivity with other antigens of the present invention. Rabbit anti-sera was generated against the most reactive fraction using the method described in Example 3A . The anti-sera was used to screen an *M. tuberculosis* Erdman strain genomic DNA expression library prepared as described above. Bacteriophage plaques expressing immunoreactive antigens were purified. Phagemid from the plaques was rescued and the nucleotide sequences of the *M. tuberculosis* clones determined.

Ten different clones were purified. Of these, one was found to be TbRa35, described above, and one was found to be the previously identified *M. tuberculosis* antigen, HSP60. Of the remaining eight clones, six (hereinafter referred to as RDIF2, RDIF5, RDIF8, RDIF10, RDIF11 and RDIF12) were found to bear some similarity to previously identified *M. tuberculosis* sequences. The determined DNA sequences for RDIF2, RDIF5, RDIF8, RDIF10 and RDIF11 are provided in SEQ ID NOS: 184-188, respectively, with the corresponding predicted amino acid sequences being provided in SEQ ID NOS: 189-193, respectively. The 5' and 3' DNA sequences for RDIF12 are provided in SEQ ID NOS: 194 and 195, respectively. No significant homologies were found to the antigen RDIF-7. The determined DNA and predicted amino acid sequences for RDIF7 are provided in SEQ ID NOS: 196 and 197, respectively. One additional clone, referred to as RDIF6 was isolated, however, this was found to be identical to RDIF5.

Recombinant RDIF6, RDIF8, RDIF10 and RDIF11 were prepared as described above. These antigens were found to stimulate cell proliferation and IFN-γ production in T cells isolated from *M. tuberculosis*-immune donors.

EXAMPLE 4

PURIFICATION AND CHARACTERIZATION OF A POLYPEPTIDE FROM TUBERCULIN PURIFIED PROTEIN DERIVATIVE

An *M. tuberculosis* polypeptide was isolated from tuberculin purified protein derivative (PPD) as follows.

PPD was prepared as published with some modification (Seibert, F. et al., Tuberculin purified protein derivative. Preparation and analyses of a large quantity for standard. The American Review of Tuberculosis 44:9-25, 1941). *M. tuberculosis* Rv strain was grown for 6 weeks in synthetic medium in roller bottles at 37°C. Bottles containing the bacterial growth were then heated to 100°C in water vapor for 3 hours. Cultures were sterile filtered using a 0.22 μ filter and the liquid phase was concentrated 20 times using a 3 kD cut-off membrane. Proteins were precipitated once with 50% ammonium sulfate solution and eight times with 25% ammonium sulfate solution. The resulting proteins (PPD) were fractionated by reverse phase liquid chromatography (RP-HPLC) using a C18 column (7.8 x 300 mM; Waters, Milford, MA) in a Biocad HPLC system (Perseptive Biosystems, Framingham, MA). Fractions were eluted from the column with a linear gradient from 0-100% buffer (0.1% TFA in acetonitrile). The flow rate was 10 ml/minute and eluent was monitored at 214 nm and 280 nm.

Six fractions were collected, dried, suspended in PBS and tested individually in *M. tuberculosis*-infected guinea pigs for induction of delayed type hypersensitivity (DTH) reaction. One fraction was found to induce a strong DTH reaction and was subsequently fractionated further by RP-HPLC on a microbore Vydac C18 column (Cat. No. 218TP5115) in a Perkin Elmer/Applied Biosystems Division Model 172 HPLC. Fractions were eluted with a linear gradient from 5-100% buffer (0.05% TFA in acetonitrile) with a flow rate of 80 μl/minute. Eluent was monitored at 215 nm. Eight fractions were collected and tested for induction of DTH in *M. tuberculosis*-infected guinea pigs. One fraction was found to induce strong DTH of about 16 mm induration. The other fractions did not induce detectable DTH. The positive fraction was submitted to SDS-PAGE gel electrophoresis and found to contain a single protein band of approximately 12 kD molecular weight.

This polypeptide, herein after referred to as DPPD, was sequenced from the amino terminal using a Perkin Elmer/Applied Biosystems Division Procise 492 protein sequencer as described above and found to have the N-terminal sequence shown in SEQ ID NO:: 124. Comparison of this sequence with known sequences in the gene bank as described above revealed no known homologies. Four cyanogen bromide fragments of DPPD were isolated and found to have the sequences shown in SEQ ID NOS: 125-128.

EXAMPLE 5

SYNTHESIS OF SYNTHETIC POLYPEPTIDES

Polypeptides may be synthesized on a Millipore 9050 peptide synthesizer using FMOC chemistry with HPTU (O-Benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate) activation. A Gly-Cys-Gly sequence may be attached to the amino terminus of the peptide to provide a method of conjugation or labeling of the peptide. Cleavage of the peptides from the solid support may be carried out using the following cleavage mixture: trifluoroacetic acid:ethanedithiol:thioanisole:water:phenol (40:1:2:2:3). After cleaving for 2 hours, the peptides may be precipitated in cold methyl-t-butyl-ether. The peptide pellets may then be dissolved in water containing 0.1% trifluoroacetic acid (TFA) and lyophilized prior to purification by C18 reverse phase HPLC. A gradient of 0-60% acetonitrile (containing 0.1% TFA) in water (containing 0.1% TFA) may be used to elute the peptides. Following lyophilization of the pure fractions, the peptides may be characterized using electrospray mass spectrometry and by amino acid analysis.

This procedure was used to synthesize a TbM-1 peptide that contains one and a half repeats of a TbM-1 sequence. The TbM-1 peptide has the sequence GCGDRSGGNLDQIRLRRDRSGGNL (SEQ ID NO: 63).

EXAMPLE 6

USE OF REPRESENTATIVE ANTIGENS FOR SERODIAGNOSIS OF TUBERCULOSIS

This Example illustrates the diagnostic properties of several representative antigens.

Assays were performed in 96-well plates were coated with 200 ng antigen diluted to 50 µL in carbonate coating buffer, pH 9.6. The wells were coated overnight at 4°C (or 2 hours at 37°C). The plate contents were then removed and the wells were blocked for 2 hours with 200 µL of PBS/1% BSA. After the blocking step, the wells were washed five

times with PBS/0.1% Tween 20™. 50 µL sera, diluted 1:100 in PBS/0.1% Tween 20™/0.1% BSA, was then added to each well and incubated for 30 minutes at room temperature. The plates were then washed again five times with PBS/0.1% Tween 20™.

The enzyme conjugate (horseradish peroxidase - Protein A, Zymed, San Francisco, CA) was then diluted 1:10,000 in PBS/0.1% Tween 20™/0.1% BSA, and 50 µL of the diluted conjugate was added to each well and incubated for 30 minutes at room temperature. Following incubation, the wells were washed five times with PBS/0.1% Tween 20™. 100 µL of tetramethylbenzidine peroxidase (TMB) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added, undiluted, and incubated for about 15 minutes. The reaction was stopped with the addition of 100 µL of 1 N H_2SO_4 to each well, and the plates were read at 450 nm.

Figure 4 shows the ELISA reactivity of two recombinant antigens isolated using method A in Example 3 (TbRa3 and TbRa9) with sera from *M. tuberculosis* positive and negative patients. The reactivity of these antigens is compared to that of bacterial lysate isolated from *M. tuberculosis* strain H37Ra (Difco, Detroit, MI). In both cases, the recombinant antigens differentiated positive from negative sera. Based on cut-off values obtained from receiver-operator curves, TbRa3 detected 56 out of 87 positive sera, and TbRa9 detected 111 out of 165 positive sera.

Figure 5 illustrates the ELISA reactivity of representative antigens isolated using method B of Example 3. The reactivity of the recombinant antigens TbH4, TbH12, Tb38-1 and the peptide TbM-1 (as described in Example 4) is compared to that of the 38 kD antigen described by Andersen and Hansen, *Infect. Immun. 57*:2481-2488, 1989. Again, all of the polypeptides tested differentiated positive from negative sera. Based on cut-off values obtained from receiver-operator curves, TbH4 detected 67 out of 126 positive sera, TbH12 detected 50 out of 125 positive sera, 38-1 detected 61 out of 101 positive sera and the TbM-1 peptide detected 25 out of 30 positive sera.

The reactivity of four antigens (TbRa3, TbRa9, TbH4 and TbH12) with sera from a group of *M. tuberculosis* infected patients with differing reactivity in the acid fast stain of sputum (Smithwick and David, *Tubercle 52*:226, 1971) was also examined, and compared

to the reactivity of *M. tuberculosis* lysate and the 38 kD antigen. The results are presented in Table 3, below:

TABLE 3

REACTIVITY OF ANTIGENS WITH SERA FROM *M. TUBERCULOSIS* PATIENTS

Patient	Acid Fast Sputum	ELISA Values					
		Lysate	38kD	TbRa9	TbH12	TbH4	TbRa3
Tb01B93I-2	++++	1.853	0.634	0.998	1.022	1.030	1.314
Tb01B93I-19	++++	2.657	2.322	0.608	0.837	1.857	2.335
Tb01B93I-8	++-	2.703	0.527	0.492	0.281	0.501	2.002
Tb01B93I-10	+++	1.665	1.301	0.685	0.216	0.448	0.458
Tb01B93I-11	+++	2.817	0.697	0.509	0.301	0.173	2.608
Tb01B93I-15	+++	1.28	0.283	0.808	0.218	1.537	0.811
Tb01B93I-16	+++	2.908	>3	0.899	0.441	0.593	1.080
Tb01B93I-25	+++	0.395	0.131	0.335	0.211	0.107	0.948
Tb01B93I-87	+++	2.653	2.432	2.282	0.977	1.221	0.857
Tb01B93I-89	+++	1.912	2.370	2.436	0.876	0.520	0.952
Tb01B94I-108	+++	1.639	0.341	0.797	0.368	0.654	0.798
Tb01B94I-201	+++	1.721	0.419	0.661	0.137	0.064	0.692
Tb01B93I-88	++	1.939	1.269	2.519	1.381	0.214	0.530
Tb01B93I-92	++	2.355	2.329	2.78	0.685	0.997	2.527
Tb01B94I-109	++	0.993	0.620	0.574	0.441	0.5	2.558
Tb01B94I-210	++	2.777	>3	0.393	0.367	1.004	1.315
Tb01B94I-224	++	2.913	0.476	0.251	1.297	1.990	0.256

Patient	Acid Fast Sputum	ELISA Values					
		Lysate	38kD	TbRa9	TbH12	TbH4	TbRa3
Tb01B93I-9	+	2.649	0.278	0.210	0.140	0.181	1.586
Tb01B93I-14	+	>3	1.538	0.282	0.291	0.549	2.880
Tb01B93I-21	+	2.645	0.739	2.499	0.783	0.536	1.770
Tb01B93I-22	+	0.714	0.451	2.082	0.285	0.269	1.159
Tb01B93I-31	+	0.956	0.490	1.019	0.812	0.176	1.293
Tb01B93I-32	--	2.261	0.786	0.668	0.273	0.535	0.405
Tb01B93I-52	--	0.658	0.114	0.434	0.330	0.273	1.140
Tb01B93I-99	--	2.118	0.584	1.62	0.119	0.977	0.729
Tb01B94I-130	--	1.349	0.224	0.86	0.282	0.383	2.146
Tb01B94I-131	--	0.685	0.324	1.173	0.059	0.118	1.431
AT4-0070	Normal	0.072	0.043	0.092	0.071	0.040	0.039
AT4-0105	Normal	0.397	0.121	0.118	0.103	0.078	0.390
3/15/94-1	Normal	0.227	0.064	0.098	0.026	0.001	0.228
4/15/93-2	Normal	0.114	0.240	0.071	0.034	0.041	0.264
5/26/94-4	Normal	0.089	0.259	0.096	0.046	0.008	0.053
5/26/94-3	Normal	0.139	0.093	0.085	0.019	0.067	0.01

Based on cut-off values obtained from receiver-operator curves, TbRa3 detected 23 out of 27 positive sera, TbRa9 detected 22 out of 27, TbH4 detected 18 out of 27 and TbH12 detected 15 out of 27. If used in combination, these four antigens would have a theoretical sensitivity of 27 out of 27, indicating that these antigens should complement each other in the serological detection of *M. tuberculosis* infection. In addition, several of the recombinant antigens detected positive sera that were not detected using the 38 kD antigen, indicating that these antigens may be complementary to the 38 kD antigen.

The reactivity of the recombinant antigen TbRa11 with sera from *M. tuberculosis* patients shown to be negative for the 38 kD antigen, as well as with sera from PPD positive and normal donors, was determined by ELISA as described above. The results are shown in Figure 6 which indicates that TbRa11, while being negative with sera from PPD positive and normal donors, detected sera that were negative with the 38 kD antigen. Of the thirteen 38 kD negative sera tested, nine were positive with TbRa11, indicating that this antigen may be reacting with a sub-group of 38 kD antigen negative sera. In contrast, in a group of 38 kD positive sera where TbRa11 was reactive, the mean OD 450 for TbRa11 was lower than that for the 38 kD antigen. The data indicate an inverse relationship between the presence of TbRa11 activity and 38 kD positivity.

The antigen TbRa2A was tested in an indirect ELISA using initially 50 µl of serum at 1:100 dilution for 30 minutes at room temperature followed by washing in PBS Tween and incubating for 30 minutes with biotinylated Protein A (Zymed, San Francisco, CA) at a 1:10,000 dilution. Following washing, 50 µl of streptavidin-horseradish peroxidase (Zymed) at 1:10,000 dilution was added and the mixture incubated for 30 minutes. After washing, the assay was developed with TMB substrate as described above. The reactivity of TbRa2A with sera from *M. tuberculosis* patients and normal donors in shown in Table 4. The mean value for reactivity of TbRa2A with sera from *M. tuberculosis* patients was 0.444 with a standard deviation of 0.309. The mean for reactivity with sera from normal donors was 0.109 with a standard deviation of 0.029. Testing of 38 kD negative sera (Figure 7) also indicated that the TbRa2A antigen was capable of detecting sera in this category.

TABLE 4

REACTIVITY OF TbRa2A WITH SERA FROM *M. TUBERCULOSIS* PATIENTS AND FROM NORMAL DONORS

Serum ID	Status	OD 450
Tb85	TB	0.680
Tb86	TB	0.450
Tb87	TB	0.263
Tb88	TB	0.275
Tb89	TB	0.403

Tb91	TB	0.393
Tb92	TB	0.401
Tb93	TB	0.232
Tb94	TB	0.333
Tb95	TB	0.435
Tb96	TB	0.284
Tb97	TB	0.320
Tb99	TB	0.328
Tb100	TB	0.817
Tb101	TB	0.607
Tb102	TB	0.191
Tb103	TB	0.228
Tb107	TB	0.324
Tb109	TB	1.572
Tb112	TB	0.338
DL4-0176	Normal	0.036
AT4-0043	Normal	0.126
AT4-0044	Normal	0.130
AT4-0052	Normal	0.135
AT4-0053	Normal	0.133
AT4-0062	Normal	0.128
AT4-0070	Normal	0.088
AT4-0091	Normal	0.108
AT4-0100	Normal	0.106
AT4-0105	Normal	0.108
AT4-0109	Normal	0.105

The reactivity of the recombinant antigen (g) (SEQ ID NO: 60) with sera from *M. tuberculosis* patients and normal donors was determined by ELISA as described above. Figure 8 shows the results of the titration of antigen (g) with four *M. tuberculosis* positive sera that were all reactive with the 38 kD antigen and with four donor sera. All four positive sera were reactive with antigen (g).

The reactivity of the recombinant antigen TbH-29 (SEQ ID NO: 137) with sera from *M. tuberculosis* patients, PPD positive donors and normal donors was determined by indirect ELISA as described above. The results are shown in Figure 9. TbH-29 detected 30 out of 60 *M. tuberculosis* sera, 2 out of 8 PPD positive sera and 2 out of 27 normal sera.

Figure 10 shows the results of ELISA tests (both direct and indirect) of the antigen TbH-33 (SEQ ID NO: 140) with sera from *M. tuberculosis* patients and from normal

donors and with a pool of sera from *M. tuberculosis* patients. The mean OD 450 was demonstrated to be higher with sera from *M. tuberculosis* patients than from normal donors, with the mean OD 450 being significantly higher in the indirect ELISA than in the direct ELISA. Figure 11 is a titration curve for the reactivity of recombinant TbH-33 with sera from *M. tuberculosis* patients and from normal donors showing an increase in OD 450 with increasing concentration of antigen.

The reactivity of the recombinant antigens RDIF6, RDIF8 and RDIF10 (SEQ ID NOS: 184-187, respectively) with sera from *M. tuberculosis* patients and normal donors was determined by ELISA as described above. RDIF6 detected 6 out of 32 *M. tuberculosis* sera and 0 out of 15 normal sera; RDIF8 detected 14 out of 32 *M. tuberculosis* sera and 0 out of 15 normal sera; and RDIF10 detected 4 out of 27 *M. tuberculosis* sera and 1 out of 15 normal sera. In addition, RDIF10 was found to detect 0 out of 5 sera from PPD-positive donors.

EXAMPLE 7

PREPARATION AND CHARACTERIZATION OF *M. TUBERCULOSIS* FUSION PROTEINS

A fusion protein containing TbRa3, the 38 kD antigen and Tb38-1 was prepared as follows.

Each of the DNA constructs TbRa3, 38 kD and Tb38-1 were modified by PCR in order to facilitate their fusion and the subsequent expression of the fusion protein TbRa3-38 kD-Tb38-1. TbRa3, 38 kD and Tb38-1 DNA was used to perform PCR using the primers PDM-64 and PDM-65 (SEQ ID NO: 141 and 142), PDM-57 and PDM-58 (SEQ ID NO: 143 and 144), and PDM-69 and PDM-60 (SEQ ID NO: 145-146), respectively. In each case, the DNA amplification was performed using 10 µl 10X Pfu buffer, 2 µl 10 mM dNTPs, 2 µl each of the PCR primers at 10 µM concentration, 81.5 µl water, 1.5 µl Pfu DNA polymerase (Stratagene, La Jolla, CA) and 1 µl DNA at either 70 ng/µl (for TbRa3) or 50 ng/µl (for 38 kD and Tb38-1). For TbRa3, denaturation at 94°C was performed for 2 min, followed by 40 cycles of 96°C for 15 sec and 72°C for 1 min, and lastly by 72°C for 4 min. For 38 kD, denaturation at 96°C was performed for 2 min, followed by 40 cycles of 96°C for 30 sec,

68°C for 15 sec and 72°C for 3 min, and finally by 72°C for 4 min. For Tb38-1 denaturation at 94°C for 2 min was followed by 10 cycles of 96°C for 15 sec, 68°C for 15 sec and 72°C for 1.5 min, 30 cycles of 96°C for 15 sec, 64°C for 15 sec and 72°C for 1.5, and finally by 72°C for 4 min.

The TbRa3 PCR fragment was digested with NdeI and EcoRI and cloned directly into pT7^L2 IL 1 vector using NdeI and EcoRI sites. The 38 kD PCR fragment was digested with Sse8387I, treated with T4 DNA polymerase to make blunt ends and then digested with EcoRI for direct cloning into the pT7^L2Ra3-1 vector which was digested with StuI and EcoRI. The 38-1 PCR fragment was digested with Eco47III and EcoRI and directly subcloned into pT7^L2Ra3/38kD-17 digested with the same enzymes. The whole fusion was then transferred to pET28b using NdeI and EcoRI sites. The fusion construct was confirmed by DNA sequencing.

The expression construct was transformed to BLR pLys S *E. coli* (Novagen, Madison, WI) and grown overnight in LB broth with kanamycin (30 μg/ml) and chloramphenicol (34 μg/ml). This culture (12 ml) was used to inoculate 500 ml 2XYT with the same antibiotics and the culture was induced with IPTG at an OD560 of 0.44 to a final concentration of 1.2 mM. Four hours post-induction, the bacteria were harvested and sonicated in 20 mM Tris (8.0), 100 mM NaCl, 0.1% DOC, 20 μg/ml Leupeptin, 20 mM PMSF followed by centrifugation at 26,000 X g. The resulting pellet was resuspended in 8 M urea, 20 mM Tris (8.0), 100 mM NaCl and bound to Pro-bond nickel resin (Invitrogen, Carlsbad, CA). The column was washed several times with the above buffer then eluted with an imidazole gradient (50 mM, 100 mM, 500 mM imidazole was added to 8 M urea, 20 mM Tris (8.0), 100 mM NaCl). The eluates containing the protein of interest were then dialyzed against 10 mM Tris (8.0).

The DNA and amino acid sequences for the resulting fusion protein (hereinafter referred to as TbRa3-38 kD-Tb38-1) are provided in SEQ ID NO: 147 and 148, respectively.

A fusion protein containing the two antigens TbH-9 and Tb38-1 (hereinafter referred to as TbH9-Tb38-1) without a hinge sequence, was prepared using a similar

procedure to that described above. The DNA sequence for the TbH9-Tb38-1 fusion protein is provided in SEQ ID NO: 151.

A fusion protein containing TbRa3, the antigen 38kD, Tb38-1 and DPEP was prepared as follows.

Each of the DNA constructs TbRa3, 38 kD and Tb38-1 were modified by PCR and cloned into vectors essentially as described above, with the primers PDM-69 (SEQ ID NO:145 and PDM-83 (SEQ ID NO: 200) being used for amplification of the Tb38-1A fragment. Tb38-1A differs from Tb38-1 by a DraI site at the 3' end of the coding region that keeps the final amino acid intact while creating a blunt restriction site that is in frame. The TbRa3/38kD/Tb38-1A fusion was then transferred to pET28b using NdeI and EcoR1 sites.

DPEP DNA was used to perform PCR using the primers PDM-84 and PDM-85 (SEQ ID NO: 201 and 202, respectively) and 1 µl DNA at 50 ng/µl. Denaturation at 94 °C was performed for 2 min, followed by 10 cycles of 96 °C for 15 sec, 68 °C for 15 sec and 72 °C for 1.5 min; 30 cycles of 96 °C for 15 sec, 64 °C for 15 sec and 72 °C for 1.5 min; and finally by 72 °C for 4 min. The DPEP PCR fragment was digested with EcoRI and Eco72I and clones directly into the pET28Ra3/38kD/38-1A construct which was digested with DraI and EcoRI. The fusion construct was confirmed to be correct by DNA sequencing. Recombinant protein was prepared as described above. The DNA and amino acid sequences for the resulting fusion protein (hereinafter referred to as TbF-2) are provided in SEQ ID NO: 203 and 204, respectively.

EXAMPLE 8

USE OF *M. TUBERCULOSIS* FUSION PROTEINS FOR SERODIAGNOSIS OF TUBERCULOSIS

The effectiveness of the fusion protein TbRa3-38 kD-Tb38-1, prepared as described above, in the serodiagnosis of tuberculosis infection was examined by ELISA.

The ELISA protocol was as described above in Example 6, with the fusion protein being coated at 200 ng/well. A panel of sera was chosen from a group of tuberculosis patients previously shown, either by ELISA or by western blot analysis, to react with each of

the three antigens individually or in combination. Such a panel enabled the dissection of the serological reactivity of the fusion protein to determine if all three epitopes functioned with the fusion protein. As shown in Table 5, all four sera that reacted with TbRa3 only were detectable with the fusion protein. Three sera that reacted only with Tb38-1 were also detectable, as were two sear that reacted with 38 kD alone. The remaining 15 sera were all positive with the fusion protein based on a cut-off in the assay of mean negatives +3 standard deviations. This data demonstrates the functional activity of all three epitopes in the fusion protein.

TABLE 5

REACTIVITY OF TRI-PEPTIDE FUSION PROTEIN WITH SERA FROM *M. TUBERCULOSIS* PATIENTS

Serum ID	Status	ELISA and/or Western Blot Reactivity with Individual proteins			Fusion recombinant OD 450	Fusion Recombinant Status
		38kd	Tb38-1	TbRa3		
01B93I-40	TB	-	-	+	0.413	+
01B93I-41	TB	-	+	+	0.392	+
01B93I-29	TB	+	-	+	2.217	+
01B93I-109	TB	+	+	+	0.522	+
01B93I-132	TB	+	+	+	0.937	+
5004	TB	+	+	+	1.098	+
15004	TB	+	+	+	2.077	+
39004	TB	+	+	+	1.675	+
68004	TB	+	+	+	2.388	+
99004	TB	-	+	+	0.607	+
107004	TB	-	+	+	0.667	+
92004	TB	+	+	+	1.070	+
97004	TB	+	-	+	1.152	+
118004	TB	+	-	+	2.694	+
173004	TB	+	+	+	3.258	+
175004	TB	+	-	+	2.514	+
274004	TB	-	-	+	3.220	+
276004	TB	-	+	-	2.991	+
282004	TB	+	-	-	0.824	+

289004	TB	-	-	+	0.848	+
308004	TB	-	+	-	3.338	+
314004	TB	-	+	-	1.362	+
317004	TB	+	-	-	0.763	+
312004	TB	-	-	+	1.079	+
D176	PPD	-	-	-	0.145	-
D162	PPD	-	-	-	0.073	-
D161	PPD	-	-	-	0.097	-
D27	PPD	-	-	-	0.082	-
A6-124	NORMAL	-	-	-	0.053	-
A6-125	NORMAL	-	-	-	0.087	-
A6-126	NORMAL	-	-	-	0.346	±
A6-127	NORMAL	-	-	-	0.064	-
A6-128	NORMAL	-	-	-	0.034	-
A6-129	NORMAL	-	-	-	0.037	-
A6-130	NORMAL	-	-	-	0.057	-
A6-131	NORMAL	-	-	-	0.054	-
A6-132	NORMAL	-	-		0.022	-
A6-133	NORMAL	-	-		0.147	-
A6-134	NORMAL	-	-	-	0.101	-
A6-135	NORMAL	-	-		0.066	-
A6-136	NORMAL	-	-		0.054	-
A6-137	NORMAL	-	-	-	0.065	-
A6-138	NORMAL	-	-	-	0.041	-
A6-139	NORMAL	-	-	-	0.103	-
A6-140	NORMAL	-	-	-	0.212	-
A6-141	NORMAL	-	-	-	0.056	-
A6-142	NORMAL	-	-	-	0.051	-

The reactivity of the fusion protein TbF-2 with sera from *M. tuberculosis*-infected patients was examined by ELISA using the protocol described above. The results of these studies (Table 6) demonstrate that all four antigens function independently in the fusion protein.

TABLE 6

REACTIVITY OF TbF-2 FUSION PROTEIN WITH TB AND NORMAL SERA

Serum ID	Status	TbF OD450	Status	TbF-2 OD450	Status	ELISA Reactivity			
						38 kD	TbRa3	Tb38-1	DPEP
B931-40	TB	0.57	+	0.321	+	-	+	-	+
B931-41	TB	0.601	+	0.396	+	+	+	+	-
B931-108	TB	0.494	+	0.404	+	+	+	±	-
B931-132	TB	1.502	+	1.202	+	+	+	+	±
5004	TB	1.805	+	1.666	+	±	±	+	-
15004	TB	2.862	+	2.468	+	+	+	+	-
39004	TB	2.443	+	1.722	+	+	+	+	-
68004	TB	2.871	+	2.575	+	+	+	+	-
99004	TB	0.691	+	0.971	+	-	±	+	-
107004	TB	0.875	+	0.732	+	-	±	+	-
92004	TB	1.632	+	1.394	+	+	+	+	-
97004	TB	1.491	+	1.979	+	+	±	-	+
118004	TB	3.182	+	3.045	+	+	±	-	-
173004	TB	3.644	+	3.578	+	+	+	+	-
175004	TB	3.332	+	2.916	+	+	+	+	-
274004	TB	3.696	+	3.716	+	-	+	-	+
276004	TB	3.243	+	2.56	+	-	-	+	-
282004	TB	1.249	+	1.234	+	+	-	-	-
289004	TB	1.373	+	1.17	+	-	±	-	-
308004	TB	3.708	+	3.355	+	-	-	+	-
314004	TB	1.663	+	1.399	+	-	-	+	-
317004	TB	1.163	+	0.92	+	+	-	+	-
312004	TB	1.709	+	1.453	+	-	+	-	-
330004	TB	0.238	-	0.461	+	-	±	-	+
451004	TB	0.18	-	0.2	-	-	-	-	-
478004	TB	0.188	-	0.469	+	-	-	+	±
410004	TB	0.384	+	2.392	+	±	-	-	+
411004	TB	0.306	+	0.878	+	-	+	-	+
421004	TB	0.357	+	1.456	+	-	+	-	+
528004	TB	0.047	-	0.196	-	-	-	-	-
A6-87	Normal	0.094	-	0.865	-	-	-	-	-
A6-88	Normal	0.214	-	0.19	-	-	-	-	-
A6-89	Normal	0.248	-	0.125	-	-	-	-	-
A6-90	Normal	0.179	-	6.206	-	-	-	-	-
A6-91	Normal	0.135	-	0.151	-	-	-	-	-
A6-92	Normal	0.004	-	0.097	-	-	-	-	-
A6-93	Normal	0.072	-	0.698	-	-	-	-	-
A6-94	Normal	0.072	-	0.064	-	-	-	-	-
A6-95	Normal	0.125	-	0.139	-	-	-	-	-
A6-96	Normal	0.121	-	0.12	-	-	-	-	-
Cut-off		0.284		0.266					

One of skill in the art will appreciate that the order of the individual antigens within the fusion protein may be changed and that comparable activity would be expected provided each of the epitopes is still functionally available. In addition, truncated forms of the proteins containing active epitopes may be used in the construction of fusion proteins.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purpose of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: COMPOUNDS AND METHODS FOR DIAGNOSIS OF TUBERCULOSIS

(iii) NUMBER OF SEQUENCES: 280

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Patentin Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
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(B) REGISTRATION NUMBER: 31,392
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 766 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear